STUDIES ON HISTIDINE FERMENTATION

KAZUMI ARAKI

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CONTENTS

Chapter I. Introduction

L-Histidine is one of the amino acids which are commonly found in protein. It was isolated from acid-hydrolyzate of sturine (a protamine of strugeon sperm) by Kossel[1] in 1896. Hedin[2] independently isolated L-histidine from protein hydrolyzates in the same year. Pauly[3] and others[4,5] provided evidence for the presence of imidazole ring and elucidated the structure, which was proved by synthesis in 1911 by Pyman.[6]

L-Histidine is required for growth in rat, dog, mouse and chick.[7,8] It is not "essential for human adults,[9] but infants require L-histidine.[10] The major demand for this amino acid is found in "transfusion solution" at present. L-Histidine is also useful as a starting material for the production of its related substances. For example, L-histamine (a powerful vasodilator) and urocanic acid (an anti-sunburn agent) can be easily produced from L-histidine on decarboxylation and deamination, respectively.[11]

Prior to the present study, L-histidine had been supplied by means of isolation from protein hydrolyzates or chemical synthesis. However, it was still one of the high-cost amino acids. The present work has aimed at the developement of a fermentation process for L-histidine production using microbial mutants.

The fermentation production of amino acids started with the discovery of an efficient L-glutamic acid producer, Corynebacterium glutamicum (synonim Micrococcus glutamicus) by Kinoshita et al.[12] in 1957. Subsequent works, however,

revealed that wild-type strains isolated from nature could accumulate only restricted kinds of amino acids (L-glutamic acid, DL-alanine, L-valine, L-glutamine and L-proline) in industrially significant amount. The main cause of this is generally ascribed to the regulation of cellular metabolism to avoid overproduction of the amino acids.[13] The important mechanisms of the regulation are through two types of regulations known as feedback inhibition[14] and repression.[15] In both cases, end product or a metabolite of the end product of the reaction sequence acts as a regulatory effector.

An auxotrophic mutant which has defect in the biosynthesis of the regulatory effector overproduces and excretes the precursors of the related metabolite of the blocked reaction when grown on a limited supply of the effector, i.e., the requisite substance. The isolation of the precursors for the biosynthesis of an amino acid has provided clues to the biosynthetic pathway for the amino acid.[16] Active attempts to utilize this phenomenon for the industrial production of amino acid were launched upto the 1950s. The first prominent result was obtained by Casid and Baldwin[17] in producing α, ε-diaminopimelic acid, a terminal intermediate for L-lysine biosynthesis, using a lysine auxotroph of Escherichia coli. Soon afterward, a process for L-ornithine (a precursor for L-arginine biosynthesis) production was developed with an arginine auxotroph of C. glutamicum by Kinoshita et al.[18]

L-Lysine production with a homoserine (or threonine plus methionine) auxotroph of C. glutamicum is the first instance of the production of the end product amino acid.[19] In this bacterium, L-lysine, L-threonine and L-methionine are formed via a

branched pathway in which L-aspartic acid is a common precursor. The reaction of the first enzyme (aspartate kinase EC 2.7.2.4) of the pathway is inhibited by the presence of both L-lysine and L-threonine, but not inhibited by each alone.[20] The genetic block at the reaction specific to L-threonine (or both L-threonine and L-methionine) and a limited supply of L-threonine to the mutant cells release the regulation and cause overproduction and accumulation of L-lysine in the culture medium.

The auxotrophic mutant is useless to produce the end product amino acid which is formed via an unbranched pathway. Because in such a case the regulatory effector is the end product itself (or the metabolite of the end product) which is desired to be produced, the block of the biosynthesis of the effector means the block of the biosynthesis of the desired product. The production of such an amino acid depends on the regulatory mutant which is obtainable as a mutant resistant to the structural analog of the amino acid. It is generally been recognized in microbial systems that a structural analog of the normal metabolite mimics the regulatory action of the metabolite and that mutations causing resistance to the analog involve a mutation causing overproduction and excretion of the metabolite or its related metabolite.[21-23] The L-threonine production is based on this phenomenon.[24,25] A mutant which was derived as α-amino- β -hydroxyvaleric acid (a threonine analog)-resistant produced a large amount of L-threonine in the culture medium. This type of the mutant derived from Brevibacterium flavum had a homoserine dehydrogenase (EC 1.1.1.3, a key enzyme for L-threonine

biosynthesis), which was altered to be insensitive to the feedback inhibition of L-threonine. The altered nature of the homoserine dehydrogenase is believed to be a main cause of the L-threonine production by this mutant.[26)]

Another fermentation process used for amino acid production is based on the microbial conversion of a precursor into the corresponding amino acid. Production of L-lysine from α, ε-diaminopimelic acid[17)] and of L-aspartic acid from fumaric acid[27,28)] may be mentioned as examples.

Nowadays, besides the amino acids described above, many amino acids including L-arginine, L-citrulline, glycine, L-homoserine, L-isoleucine, L-leucine, L-methionine, L-serine, L-phenylalanine, L-tyrosine and L-tryptophan can be produced through the fermentation processes described above and the combination thereof.[20)]

The biosynthetic pathway of L-histidine has been clarified by Ames and others[29)] largely with the mutants of Salmonella typhimurium. It consists of ten enzyme reactions as shown in Fig. 1. The first step in the reaction sequence is catalyzed by a regulatory enzyme, phosphoribosyl-ATP pyrophosphorylase (EC 4.1.2c), which is inhibited by L-histidine, the end product of the sequence. The presence of excess L-histidine in the culture medium represses the synthesis of the entire sequence of the L-histidine-forming enzymes.

Under these circumstances, the author incidentally found the accumulation of L-histidinol (the terminal intermediate for L-histidine biosynthesis) and imidazoleglycerol (the dephosphorylated product of imidazoleglycerol phosphate, the 6th intermediate for L-histidine biosynthesis) by histidine

auxotrophs of bacteria. Then, the culture condition for the production of the imidazoles and the microbial conversion of the imidazoles into L-histidine were investigated. Chapters II and III deal with such two-steps processes for L-histidine production.

Chapter IV deals with the direct production of L-histidine from sugar and ammonium salts as starting materials. It is based on the finding that histidine analog-resistant mutants of *C. glutamicum* accumulate a large amount of L-histidine in the culture medium.

Independently of the investigations described here, Kubota *et al.*[30-32] have reported on L-histidine production by the mutants of *B. flavum*.

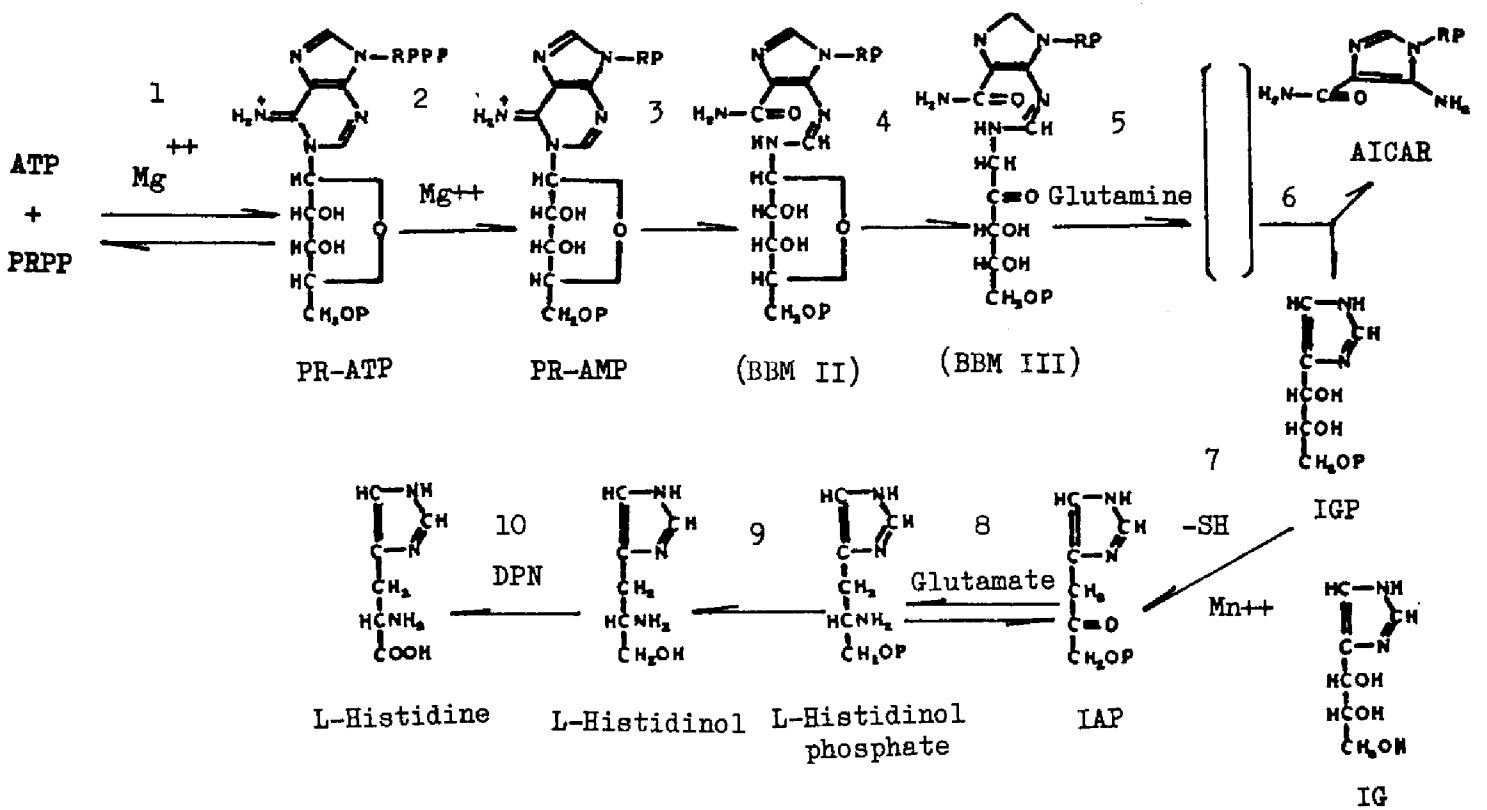
ATP
+
PRPP
Mg++
1
2
Mg++
3
4
5
Glutamine
6
AICAR
PR-ATP
PR-AMP
(BBM II)
(BBM III)
IGP
7
-SH
Mn++
IAP
8
Glutamate
9
10
DPN
L-Histidine
L-Histidinol
L-Histidinol phosphate
IG

Fig. 1. The Pathway of L-Histidine Biosynthesis in *Salmonella typhimurium*

The abbreviations used are: ATP, adenosine 5'-triphosphate; PRPP, 5-phosphoribosyl-1-pyrophosphate; PR-AMP and PR-ATP, N-1-(5'-phosphoribosyl) adenosine mono- and tri-phosphate; BBM II, N-(5'phospho-D-ribosylformimino)-5-amino-1-(5''-phosphoribosyl)-4-imidazolyl carboxamide; BBM III, N-(5'-phospho-D-1' -ribulosylformimino)-5-amino-1-(5''-phosphoribosyl) -4-imidazolecarboxamide; IGP, D-*erythro*-imidazoleglycerol phosphate; IAP, imidazoleacetol phosphate; AICAR, 5-amino-1-(5'-phosphoribosyl)-4-imidazolecarboxamide.

Enzymes: 1, PR-ATP pyrophosphorylase; 2, PR-ATP pyrophosphohydlase; 3, PR-AMP 1,6-cyclohydrolase; 4, BBM II ketoisomerase; 5, Amidotransferase; 6, Cyclase; 7, IGP dehydratase; 8, Histidinolphosphate aminotransferase; 9, Histidinolphosphate phosphatase; 10, Histidinol dehydrogenase.

Chapter II. L-Histidine Production from L-Histidinol

Section 1. L-Histidinol Production with a Histidine Auxotroph of *Corynebacterium glutamicum* and Conversion of the L-Histidinol into L-Histidine by an *Escherichia coli* Strain

INTRODUCTION

Vogel *et al.*[33] 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[34~37] and bacterial[38~41] histidine auxotrophs, and it has been known that L-histidinol is a terminal member of a sequence of imidazole intermediates on the L-histidine biosynthetic pathway (Fig. 1 in Chapter I).[29]

Recently, Kubota *et al.*[30] reported that more than 9 mg/ml of L-histidinol (as dihydrochloride) was accumulated in the culture medium of a histidine auxotroph of *Brevibacterium flavum*. These investigators also described that a considerable amount of L-histidine was accumulated in the culture medium of various kinds of microorganisms when L-histidinol was supplemented to the medium.

During the course of studies on the amino acid production with microbial auxotrophic mutants, the author found the accumulation of L-histidinol in the culture medium of a histidine auxotroph of *Corynebacterium glutamicum*. This chapter deals with the L-histidinol production by this mutant and the

microbial conversion of the produced L-histidinol into L-histidine with other microorganisms. A part of data on the subject has been described in patent descriptions.[42,43]

MATERIALS AND METHODS

Microorganisms. A histidine auxotroph, C. glutamicum KY-10234, derived from a wild-type strain ATCC 13761 by the mutagenic treatment with N-methyl-N'-nitro-N-nitrosoguanidine was mainly used in the present study. This mutant strain has been deposited to The American Type Culture Collection with an accession number of ATCC 21339. All the microorganisms employed for the conversion test of L-histidinol into L-histidine were stock cultures of Tokyo Research Laboratory of Kyowa Hakko Kogyo Co., Ltd. Salmonella typhimurium hisG46 was a gift of Dr. B.N.Ames.[46]

Culture method for L-histidinol production. Forty milliliters of a seed medium in a 250-ml Erlenmeyer flask was inoculated with the cells of microorganisms grown on a bouillon agar slant, and incubated at 28°C on a rotary shaker operated at 220 rpm. After incubation for 24 hr, 1 ml of the seed culture was transferred into a flask of the same type 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 basal compositions of the seed medium and the fermentation medium are presented in Table I. L-Histidine and several natural nutrients were added to the fermentation medium at variable levels. In the experiment shown in Table VI, $FeSO_4 \cdot 7H_2O$ and $MnSO_4 \cdot 4H_2O$ were also added to the medium.

The fermentation test shown in Fig. 2 was carried out in a 5-liter jar fermentor principally according to the flask test described above. The details of the test are described in the legend of the figure.

Table I. Basal Composition of Media

Ingredients (Amount in 100 ml)	Seed medium	Fermentation medium
Cane molasses (g*)		15
Glucose(g)	4	
Peptone(g)	2	
Yeast extract(g)	0.5	
Urea(g)	0.3	
$(NH_4)_2SO_4$(g)		2 or 3
KH_2PO_4(g)	0.15	0.15
K_2HPO_4(g)	0.05	0.05
$MgSO_4 \cdot 7H_2O$	0.05	0.05
Biotin(μg)	3	
$CaCO_3$(g)		3

*) As glucose concentration.
The hydrogen ion concentration of the media was adjusted to pH 7.4 with NH_4OH, then the media were sterilized at 120°C for 10^4 min.

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 KY-10234 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 aliguot of the cell-suspension was trans-

ferred into the flask containing 5-days culture of C. glutamicum KY-10234 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 6N 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 ion concentration in the medium was adjusted by adding an appropriate amount of $(NH_4)_2SO_4$ to make the final concentration 3% as $(NH_4)_2SO_4$, when these acid-hydrolyzates were used at variable levels.

Analysis. L-Histidine and L-histidinol were determined as follows: An aliquot of the culture broth was developed in paperchromatography with n-propanol-0.2N NH_4OH (3:1 by volume) as solvent. Though both of L-histidine and L-histidinol are not fluorescent, they could be located on the paperchromatogram under UV-light, by consulting the locations of the fluorescent spots of other unidentified substances being constantly contained in the culture broth. The areas of these imidazoles were cut out and they were eluted therefrom with each of 5-ml water at 60°C for 30 min. The resulting solutions were, after cooling, applied to the colorimetric assay of Mcpherson.[44] The amount of L-histidine and L-histidinol was calculated from calibration curves prepared with authentic samples. The growth of the microorganisms was measured

by reading the absobancy at 660 nm of the culture in a cuvette with 10-mm light path, using a Hitachi Colorimeter Model 101 after dissolving the $CaCO_3$ in the medium and 100-fold dilution.

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, KY-10234, 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-0.2N NH_4OH (3 : 1 by volume) as solvent was extracted with hot water. After concentration in vacuo, the extract was rechromatogramed 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 solvent systems with authentic L-histidinol, as shown in Table II, and supported the growth of S. typhimurium hisG46, which is known to respond to L-histidine and L-histidinol but not to D-isomers of these substances, as effectively as authentic L-histidinol. From these results, the author concluded the substance produced by C. glutamicum KY-10234 as L-histidinol.

Table II. Paperchromatographic Identification of the Substance Produced by C. glutamicum KY-10234 as L-Histidinol

Solvent system*	Substance produced by KY-10234	Authentic L-histidinol
A	0.72	0.72
B	0.81	0.82
C	0.47	0.49
D	0.37	0.37

*) Solvent system (ratio by volume): A, n-propanol-0.2N NH_4OH(3:1); B, isobutyric acid-28% NH_4OH(100:20); C, n-butanol saturated with 2N NH_4OH; D, n-propanol-N acetic acid (3:1).

Rf-values of the spots located with diazosulfanilic acid-reaction are given.

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

In the fermentative production of amino acids with bacterial auxotrophs, supplying of the required substance in a suboptimal level for growth gives the maximal yield of the amino acids.[20] Because KY-10234 requires L-histidine for growth, 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, the concentration suboptimal for the growth of this mutant. 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 KY-10234 during the fermentation. 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

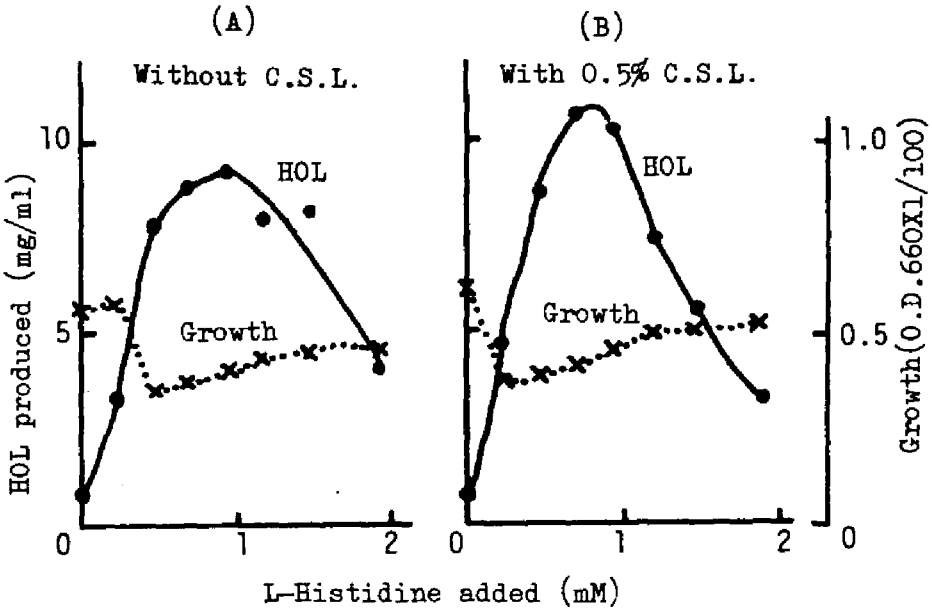

Fig. 1 Effect of L-Histidine on L-Histidinol Production by C. glutamicum KY-10234

HOL: L-Histidinol·2HCl. C.S.L: Corn steep liquor. The fermentation medium contained 3% $(NH_4)_2SO_4$.

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.

3. Effect of natural nutrients on L-histidinol production.

In the fermentative production of amino acids 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 KY-10234 was investigated. As shown in Table III, 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 significantly stimulated the growth of the mutant (Table IV). 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).

Table III Effect of Acid-hydrolyzate of S.B.M.[a] and of C. glutamicum Cells on the L-Histidinol Production by C. glutamicum KY-10234

Natural nutrients added (%)[b]		L-Histidinol produced[c] (mg/ml)	Growth (O.D.660X1/100)
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 C. glutamicum cells	(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.

Table IV. Effect of Meat Extract, Peptone and Corn Steep Liquor on L-Histidinol Production by C. glutamicum KY-10234, When Added in Combination with Acid-hydrolyzate of S.B.M. and of the Cells of C. glutamicum to the Medium

	Addition (%, W/V)		L-Histidinol produced (mg/ml) a)	Growth (O.D.660X1/100)
I	Meat extract	(1.5)	trace	0.52
		(0.75)	trace	0.46
	Corn steep liquor	(3.0)	7.4	0.35
		(1.5)	7.4	0.35
	Peptone	(1.5)	6.1	0.46
		(0.75)	6.4	0.35

	Addition (%, W/V)		L-Histidinol produced (mg/ml)[a]	Growth (O.D.660X1/100)
II	Meat extract	(1.5)	trace	0.47
		(0.75)	trace	0.53
	Corn steep liquor	(3.0)	10.1	0.47
		(1.5)	9.3	0.40
	Peptone	(1.5)	7.9	0.50
		(0.75)	7.6	0.40

a) As dihydrochloride.

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

4. L-Histidinol production in a jar fermentor.

The time course of L-histidinol fermentation by KY-10234 was investigated in a 5-liters jar fermentor with a medium containing 15% (as glucose concentration) cane molasses, 3% $(NH_4)_2SO_4$, 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 arrow in Fig. 2. After the addition of meat extract, the growth level slowly increased until 107 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.[30] 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 containg 0.5% L-histidinol dihydrochloride.

To provide more direct method for L-histidine production, the author sought the microorganisms which were capable of converting the L-histidinol accumulated by KY-10234 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 ammoniagenes 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 KY-10234, in which 6 ~ 7 mg/ml of L-histidinol (as dihydrochloride) had 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 for the identification of L-histidinol. The results on the paperchromatographic analysis are presented in Table V.

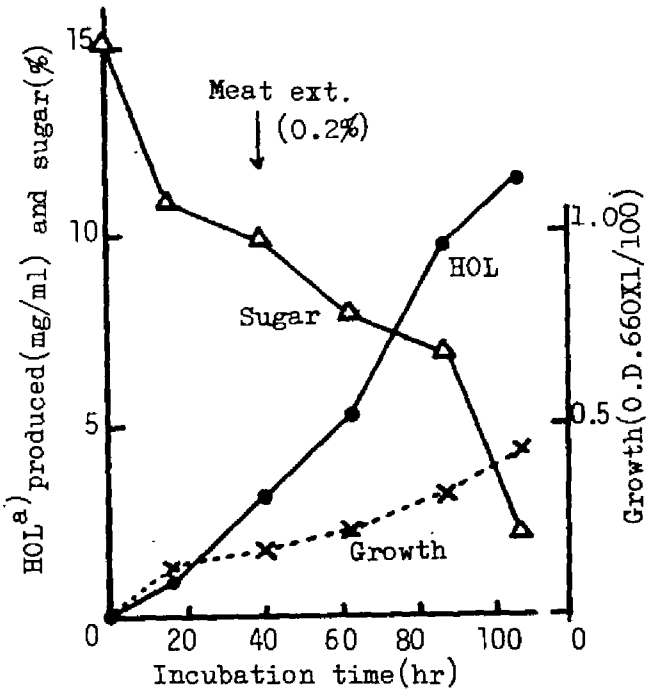

Fig. 2 L-Histidinol Production by C. glutamicum KY-10234 in a Jar Fermentor

a) L-Histidinol·2HCl

Fermentation was carried out in a 5-liters jar fermentor containing 3 liters of the fermentation medium supplemented with 1.5% (with respect to dry mater 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 NH_4OH during fermentation.

Table V. Paperchromatographic Identification of L-Histidine Formed by E. coli KY-3592 from the L-Histidinol Produced by C. glutamicum KY-10234

Solvent system[a)]	L-Histidine formed by E. coli 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-0.2N NH_4OH (3:1); B, n- propanol-N acetic acid (3:1); C, n-butanol-acetic acid-water (5:2:2); D, methylethylketone-n-butanol-28% NH_4OH-water (100:100:5:60); E, phenol-water (3:1).

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

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 VI).

Table VI. Conversion of L-Histidinol into L-Histidine by *E. coli* KY-3592

Inoculum size of *E. coli* KY-3592 (ml)	Addition(%)	L-Histidine produced (mg/ml)	Residual L-histidinol (mg/ml)[a]
0	None	0	7.3
0.5		1.9	5.5
1.0		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_3COONH_4(0.3)	2.5	5.4

C. glutamicum KY-10234 was cultured for 5 days in 7 seperate 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 fed with urea or CH_3COONH_4 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% $FeSO_4 \cdot 7H_2O$ and 0.02% $MnSO_4 \cdot 4H_2O$; the concentration of $(NH_4)_2SO_4$ was 2%.

a) As dihydrochloride.

DISCUSSION

A histidine auxotroph of *C. glutamicum*, KY-10234 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 L-histidine biosynthetic pathway of *C. glutamicum* is identical with that of other microorganisms,[29] 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.

Cultivation of E. coli KY-3592 by mixing with a later phase-culture of C. glutamicum KY-10234 resulted in the reduction of L-histidinol, which had been accumulated by KY-10234 in the culture, and the accumulation of 4 mg/ml of L-histidine. Further work is required to improve the yield of L-histidine in the two-steps method for the L-histidine production described here.

SUMMARY

A histidine auxotroph of Corynebacterium glutamicum was found to accumulate L-histidinol in the culture medium. The accumulation of it reached a level of 10 ~ 11 mg as dihydrochloride per ml with a cane molasses medium containing 15% sugar (as glucose), 2 ~ 3% $(NH_4)_2SO_4$ 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.

Section 2. Biochemical Characterization of Histidine Auxotrophs of *Corynebacterium glutamicum* and Defect of Histidinol Dehydrogenase in *C. glutamicum* KY-10234

INTRODUCTION

Vogel *et al.*[33] isolated L-histidinol from a histidine auxotroph of *Escherichia coli*. Subsequently, Ames *et al.*[34] found the accumulation of imidazoleglycerol(IG), imidazoleacetol (IA), L-histidinol and their phosphate esters by histidine auxotrophs of *Neurospora* and *Penicillium*. Of these imidazoles accumulated, only L-histidinol supported the growth of mutants blocked at earlier reactions for L-histidine biosynthesis. The other imidazoles were considered to be either impermeable to the cell membrane (e.g. phosphate esters) or not the actual precursors of L-histidine (e.g. IG and IA). These findings provided clues to the L-histidine biosynthetic pathway (Fig. 1 in Chapter I), the details of which were largely derived from genetic and enzymic studies on *Salmonella typhimurium*.[29] The pathway is considered to be identical with that of other microorganisms so far as investigated.

In the present section, the author describes a paperchromatographic analysis of diazosulfanilic acid reaction-positive imidazoles accumulated by histidine auxotrophs of *Corynebacterium glutamicum*, and correlation between the imidazole-accumulation and the defect in L-histidine biosynthetic enzymes. This study was undertaken to obtain some informations on the histidine pathway in *C. glutamicum* for a clarification of the

mechanism of the L-histidinol production by C. glutamicum KY-10234 described in the preceding section and the mechanism of L-histidine production which will be dealt in Chapter IV.

MATERIALS AND METHODS

Microorganisms. Wild-type C. glutamicum strains, ATCC 13032, ATCC 13761 and KY-9005, and their histidine auxotrophic derivatives were mainly used in this study. KY-9080, KY-10234 (ATCC 21339) and KY-9105 are histidine auxotrophs which were derived from ATCC 13032, ATCC 13761 and KY-9005, respectively, KY-9498 is a histidine-homoserine double auxotroph derived from ATCC 13287 (hom.) which is originated from ATCC 13032. S. typhimurium hisG46hisO1242[46] was a gift of Dr. B. N. Ames.

Culture method for imidazole accumulation. Histidine auxotrophs of C. glutamicum were cultured in a similar manner as that described for the L-histidinol production (Section 1 of this chapter), except that the fermentation was carried out for 4 days in a 250-ml Erlenmeyer flask settled with a baffling plate and containing 20 ml of a fermentation medium with following composition. Molasses medium: cane molasses 10g (as glucose), urea 0.3 g, $(NH_4)_2SO_4$ 4 g, KH_2PO_4 0.15 g, K_2HPO_4 0.05 g, $MgSO_4 \cdot 7H_2O$ 0.05 g, meat extract 0.5 g and $CaCO_3$ 3 g per 100 ml. Glucose medium : glucose 10 g, $(NH_4)_2SO_4$ 2 g, KH_2PO_4 0.15 g, K_2HPO_4 0.05 g, $MgSO_4 \cdot 7H_2O$ 0.05 g, $FeSO_4 \cdot 7H_2O$ 0.004 g, $MnSO_4 \cdot 4H_2O$ 0.004 g, biotin 10 µg, thiamine·HCl 500 µg, natural nutrients 0 ~ 2 g, L-histidine 0 ~ 100 mg, L-homoserine 0 or 20 mg, and $CaCO_3$ 3 g per 100 ml.

Culture method for harvesting microbial cells. S. typhimurium hisG46 hisO1242 cells grown overnight at 37°C on a bouillon agar slant (containing 6 - 7 ml of the medium in a 20 mm x 200 mm test tube) were suspended in 10 ml of water. The suspension (0.5 ml) was transferred into a 300-ml Erlenmeyer flask with 100 ml of a growth medium and incubated at 36°C for 16~20 hr on a rotary shaker operated at 210 rpm. The optical density of the culture, measured after five-fold dilution using a Hitachi Colorimeter Model 101, was 0.20~0.23 (as O.D. 660 nm x 1/5). The growth medium was the one of Vogel and Brenner[47] modified by supplementing with 0.16 mM L-histidine. C. glutamicum strains were grown at 30°C in the 300-ml flask containg 40 ml of a growth medium, in a similar manner as that described above. In this case, the growth rate varied with every strain used. Therefore, the inoculum size was adjusted to obtain the final growth level of 0.05~0.15 as the absorbancy measured after dissolving the $CaCO_3$ in the medium with HCl and 40-fold dilution by the above-described method. The growth medium contained glucose 5 g, $(NH_4)_2SO_4$ 0.5 g, urea 0.1 g, KH_2PO_4 0.1 g, K_2HPO_4 0.3 g, $MgSO_4 \cdot 7H_2O$ 0.01 g, $CaCl_2 \cdot 2H_2O$ 0.001 g, $MnCl_2 \cdot 4H_2O$ 0.0007 g, $FeSO_4 \cdot 7H_2O$ 0.001 g, NaCl 0.01 g, biotin 10 μg, thiamine·HCl 100 μg and $CaCO_3$ 2 g per 100 ml. In the case of histidine auxotrophs, the cells were grown on 0.052 mM L-histidine. The cells of KY-9498, a histidine-homoserine double auxotroph, were grown on 1.67 mM L-homoserine in addition to L-histidine.

Preparation of enzyme extract. The dialyzed cell-free extracts used in the present study were prepared according to the method of Ames et al.[48] with a slight modification. Cells of S. typhimurium hisG46his01242 were collected by centrifugation from 200 ml in total of the culture broth, sonicated in 5 ml of the triethanolamine buffer for 10 min at 80 KW, using a Kubota Ultrasonic Generator Model KMS-250. In the case of C. glutamicum strains, the cells collected by centrifugation were suspended in 7~10 ml of the buffer to make the cell concentration 50~60 mg (as dry weight) per ml, and sonicated for 30 min by the procedure described above. The sonicates were centrifuged for 30 min at 12,000 x g. The enzyme preparations contained protein in the concentration of 8~12 mg/ml and 8~15 mg/ml with the S. typhimurium mutant and C. glutamicum strains, respectively.

Enzyme assay. Histidinolphosphate (HP) aminotransferase (L-histidinolphosphate : 2-oxoglutarate aminotransferase, EC 2.6.1.9) was assayed by the method similar to that described by Ames et al.[48] for S. typhimurium enzyme, except that the enzyme was pre-incubated in the presence of 40 μM pyridoxal phosphate and the assay was scaled up to 1.5 times the original scale. The reaction mixture was read against the blank (missing HP) at 295 nm. A control (missing enzyme, ± HP) was assayed for each group of reactions and an appropriate correction was made for the assays. HP phosphatase (L-histidinolphosphate phosphohydlase, EC 3.1.3.15) was assayed by the method of Ames et al.[48] on 1.5 times the original scale, but the reaction mixture was incubated for 60 min after the termination of the reaction by the addition of ascorbic acid molybdate reagent.

Histidinol dehydrogenase [L-histidinol : nicotinamide adenine dinucleotide(NAD) oxidoreductase, EC 1.1.1.23] was assayed by the method of Martin et al.[49] on 1.5 times the original scale. The reaction mixture was read at 520 nm after two-fold dilution with 0.2 M triethanolamine-HCl buffer (original pH, 8.6) added with HCl to make final concentration of 0.117 N.

Protein determination. Protein was determined by the Folin method[50] with bovin albumin as the standard. The interference of the triethanolamine buffer, against which the enzyme extracts were dialyzed, was corrected according to the method of Ames et al.[48]

Reagents. L-Histidine·HCl, L-homoserine, nicotinamidee adenine dinucleotide phosphate (NADP), α-ketoglutarate and imidazoleglycerol·HCl were products of Kyowa Hakko Kogyo Co., Ltd. L-Histidinol·2HCl, NAD, and 5-amino-4-imidazolecarboxamide (AIC)·HCl were purchased from Sigma Chem. Co. L-Histidinol phosphate (HP)·$2H_2O$ or pyridoxal phosphate was purchased from Cyclo Chem. or Nakarai Chem. Ltd., respectively.

RESULTS

1. Accumulation of imidazoles.

Accumulation of imidazoles by histidine auxotrophs of C. glutamicum was investigated. First, molasses medium was used for the cultivation of 14 histidine auxotrophs including 10 histidine auxotrophs, two histidine-adenine double auxotrophs,

one histidine-lysine double auxotroph and one histidine-homoserine double auxotroph. Paperchromatographic analysis of culture broth obtained revealed that strains KY-9080 (his), KY-9105 (his) and KY-9498 (hom., his) accumulated AIC, IG and IA, respectively. AIC and IG were identified on the basis of Rf-values and colours of the spots of the imidazoles located by diazosulfanilic acid-reaction on paperchromatograms, in comparison with authentic samples.[38]

Because authentic IA was not available, the imidazole accumulated by KY-9498 was identified as IA by the paperchromatographic method as follows : unlike IG, L-histidinol and L-histidine, the imidazole could be located with 2,4-dinitrophenylhydrazine reagent, suggesting the imidazole has carbonyl group. The red spot of the imidazole located with diazosulfanilic acid reagent changed to a green spot, when a chromatogram was sprayed with copper sulfate solution by the method described by Ames and Mitchell[51] for the detection of IA.

Moreover, as shown in Table I, this imidazole and IG migrated in a similar relation each other as that between IA and IG as described in previous papers.[34,38]

Table I. Paperchromatographic Analysis of an Imidazole Produced by a Histidine Auxotroph, C. glutamicum KY-9498

Solvent system[a)]	Rf-values obtained		Rf-values described in previous paper[34,38)]	
	Imidazole produced by KY-9498	Authentic IG	IA	IG
A	0.44	0.38	0.54[b)]	0.42[b)]
B	0.32	0.36	0.45[c)]	0.46[c)]
C	0.95	0.74	0.73[b)]	0.63[b)]

a) Solvent system (ratio by volume): A, n-propanol-0.2N NH_4OH(3:1); B, n-propanol-N acetic acid(3:1); C, methanol-chloroform-10% formic acid(3:3:1).

b) and c) See Ref. 38) and 34), respectively.

Subsequently, the three histidine auxotrophs described above and a known histidine auxotrophic L-histidinol-producer, KY-10234 were cultured in the glucose medium supplemented with various substances required by the mutants used. The amount and the kind of imidazoles accumulated by each mutant were variable with the medium employed. Table II shows the culture conditions, i.e., the substances which were favourable for the accumulation of each imidazole, and the imidazole-accumulation under the favourable culture conditions. As can be seen in the table, all the mutants tested produced unidentified diazosulfanilic acid reaction-positive substance(s) which migrated with low Rf-value(s) (0.08 ~ 0.12) in paperchromatography with n-propanol-0.2 N NH_4OH(3 : 1 by volume)

as solvent. Besides this substance, following imidazoles were produced by each mutant : AIC and IG by KY-9080 ; IG by KY-9105 ; IA and IG by KY-9498 ; histidinol by KY-10234. Assuming that the L-histidine biosynthetic sequence in C. glutamicum was identical with that in other microorganisms (Fig. 1 in Chapter I), it was reasonable to expect that these mutants had certain defect in the following histidine enzymes : imidazoleglycerol phosphate dehydratase (EC 4.2.1.19) in KY-9080 and KY-9105 ; HP aminotransferase in KY-9498 ; histidinol dehydrogenase in KY-10234.

To confirm this expectation, the specific activities of the aminotransferase and the dehydrogenase, and HP phosphatase in the extracts from these mutants were compared with those in the extracts from their parent wild-type strains. Imidazoleglycerol phosphate dehydratase activity was not determined, because a substrate for the reaction, imidazoleglycerol phosphate, was not available.

2. Aminotransferase.

HP aminotransferase reaction was preliminarily investigated with the extract from a wild-type strain KY-9005. The reaction required α-ketoglutarate and pyridoxal phosphate, as shown in Table III. The response was linear to the enzyme concentrations up to the absorbancy of 0.2, as shown in Fig. 1. With the complete assay system, the specific activities of the aminotransferase in the extracts from 4 histidine auxotrophs listed in Table II were compared with those in the extracts from their parent wild-type strains. As shown in Table IV,

Table II. Accumulation of Imidazoles by Histidine Auxotrophs of C. glutamicum in Their Culture Broths

Strain No.	Imidazoles accumulated					Substance(%) which gave the high yield of imidazoles when added to the media
	X[a)]	AIC	IG	IA	Histidinol	
KY-9080	+	+	±	-	-	ME(2) or His(0.025) for X; ME(2) for AIC.
KY-9105	++	-	+++	-	-	NA(1) or His(0.0062) for X; PT(1~2), ME(1~2), CA(2) or His(0.025) for IG.
KY-9498	+++	-	+	+++	-	PT(1~2), NA(1~2), CA(1~2), YE(1~2) or His(0.0062) for X; PT(2), NA(1), CA(1) or His(0.0062) for IA; His(0.025) for IG.
KY-10234	++	-	-	-	++++	PT(2) or NA(2) for X; PT(2), ME(1~2), NA(2), CA(2) or His(0.0125~0.025) for histidinol.

Histidine auxotrophs of C. glutamicum were cultured in the glucose medium supplemented with each of 1 and 2 % of natural nutrients such as peptone(PT), meat extract (ME), NZ-amine (NA), casamino acids(CA) and yeast extract(YE), or 0.0062~0.1% of L-histidine monohydrochloride(His). Marks ++++ , +++ , ++ , + , + and ± show the colour density of the spots of each imidazole located with diazosulfanilic acid-reaction after paperchromatographic separation in n-propanol-0.2N NH_4OH(3:1 by volume) as solvent, in this decreasing order. The mark, ++++ , on imidazoleglycerol corresponds to 6.2 mg/ml of this imidazole determined by periodate oxidation method.[60] a) Unidentified imidazole(s) described in the text.

the specific activity of the enzyme extracted from KY-9498, an IA-producing mutant, was significantly lower than that of its parent strain ATCC 13032 and other strains tested.

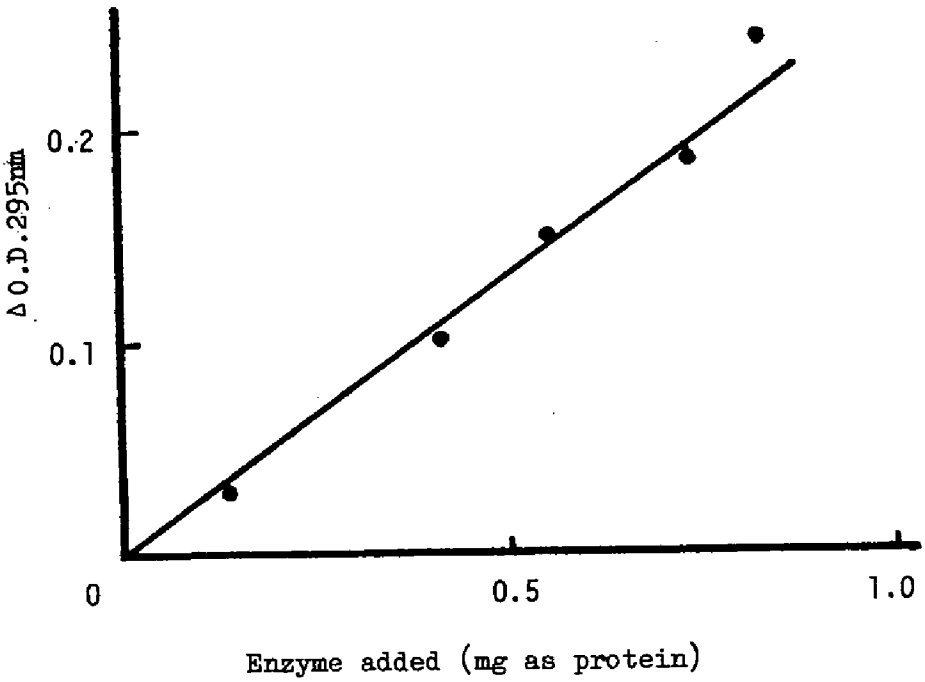

Fig. 1 Dependence of Histidinolphosphate Aminotransferase Activity on Enzyme Concentration

Extract from C. glutamicum KY-9005(wild) was used as the enzyme preparation.

Table III. Requirements for the Reaction of Histidinolphosphate Aminotransferase of C. glutamicum

Omission	ΔO.D. 295 nm
None	0.177
α-Ketoglutarate	0.000
Enzyme	-0.011
Pyridoxal phosphate	0.002

The extract from C. glutamicum KY-9005 (0.83 mg as protein) was used as the enzyme preparation.

Table IV. The Specific Activity of Three Enzymes of L-Histidine Biosynthesis in Wild-type Strains and Histidine Auxotrophs of C. glutamicum

Strain No.(Marker)	Histidinol phosphate aminotransferase (ΔOD295/20 min/mg protein)	Histidinol phosphate phosphatase (ΔOD820/30 min/mg protein)	Histidinol dehydrogenase (ΔOD520/20 min/mg protein)
ATCC 13032(wild)	0.29	0.0	1.9
KY-9498 (hom.,his)	0.03	1.5~1.9	0.2~0.3
KY-9080 (his)	0.22	2.8~3.1	0.8
ATCC 13761 (wild)	0.14	0.05	1.9~2.1
KY-10234 (his)	0.10	1.1~1.7	0.0
KY-9005 (wild)	0.22	0.05	2.0~2.2
KY-9105 (his)	0.25	0.8~1.3	1.9~2.1

3. Phosphatase.

The assay used here for HP phosphatase is based on the determination of inorganic phosphate released by the reaction using ascorbic acid molybdate reagent.[52] In this case, a little enzyme is used to avoid the adsorption of the phosphate to the precipitate of protein.[48] Though the phosphatase activity was tried to determined for the extracts from wild-type strains, ATCC 13032, ATCC 13761 and KY-9005, it could be hardly detected within the range of enzyme concentrations giving no precipitate, as shown in Table IV. Even when hydrogen ion concentration of the reaction mixture was varied between pH 5.2 ~ 7.0 with 0.2 M tris(hydroxymethyl)aminomethane-maleate-NaOH buffer and between pH 7.0 ~ 9.0 with 0.2 M triethanolamine-HCl buffer, no increase in activity was noted. The extract from ATCC 13761 did not extensively interfere with the reaction of the phosphatase in the extract from S. typhimurium hisG46his01242, when the extracts from both microorganisms were used in combination (Table V). Undialyzed extracts also exhibited no activity. On the other hand, extracts from all the histidine auxotrophs grown on 0.052 mM L-histidine exhibited phosphatase activity, though the activity was much lower than that from the above S. typhimurium mutant. The enzyme activity in the extracts from C. glutamicum mutants was assayed in the enzyme concentration which gave the absorbancy below 0.3, because the response was linear to the enzyme concentrations upto this absorbancy.

Table V. Effect of the Extract from C. glutamicum on the Reaction of Histidinolphosphate Phosphatase in S. typhimurium

Source of enzyme (Strain No.)	Enzyme added (mg as protein)	Phosphatase activity (ΔO.D. 820 nm)
C. glutamicum ATCC 13761	0.059	0.00
S. typhimurium hisG46his01242	0.067	0.87
C. glutamicum ATCC 13761	0.059	0.75
S. typhimurium hisG46his01242	0.067	

4. Dehydrogenase.

Histidinol dehydrogenase reaction was preliminarily determined with the extract from a wild-strain ATCC 13761. The reaction required NAD and L-histidinol, as shown in Table VI. NADP and HP could not substitute for these compounds, respectively. The response was linear to the enzyme concentrations upto the absorbancy of 0.9, as shown in Fig. 2. As shown in Table IV, a histidinol-producing mutant, KY-10234 had a defect in this enzyme. The specific activity in the extract from KY-9498, an IA-producing mutant, was remarkably lower than that from its parent wild-type strain ATCC 13032.

Table VI. Requirements for the Reaction of Histidinol Dehydrogenase of C. glutamicum

Omission	Addition	ΔO.D. 520 nm
None	None	0.780
NAD	None	0.051
NAD	NADP	0.033
Histidinol	Histidinol phosphate	0.070

Enzyme extract from a wild-type strain C. glutamicum ATCC 13761 (0.62 mg as protein) was used as the enzyme preparation.

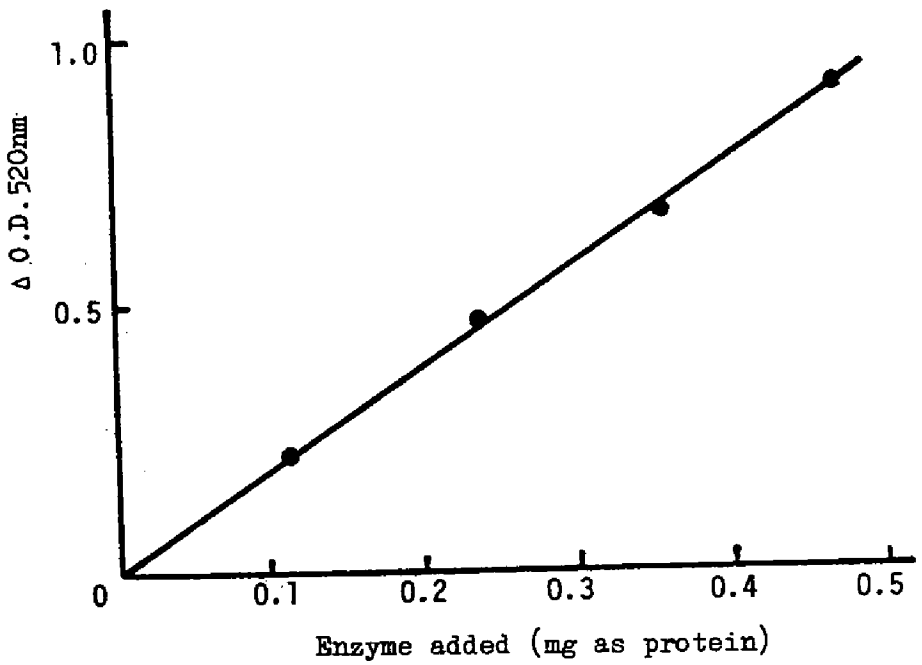

Fig. 2 Dependence of Histidinol Dehydrogenase Activity on Enzyme Concentration

Extract from C. glutamicum ATCC 13761 was used as the enzyme preparation.

DISCUSSION

It was proved that the histidine auxotrophy of the L-histidinol producer C. glutamicum KY-10234 is due to the defect in histidinol dehydrogenase. As will be described in the following chapter, the first enzyme of the histidine pathway, phosphoribosyl-ATP pyrophosphorylase, of C. glutamicum is subject to the feedback inhibition of L-histidine. The formation of the first enzyme is under the repression control of L-histidine. The L-histidinol production by KY-10234 is reasonably explained from these facts : the lack of the feedback inhibition and repression control on the histidine pathway due to the L-histidine-limitation in the medium causes the overproduction of L-histidinol and the metabolism of the produced L-histidinol is intercepted by the block at histidinol dehydrogenase, then the imidazole excretes into the medium. The accumulation of IA by KY-9498 is similarly explained in terms of the defect in HP aminotransferase and the lack of the feedback regulations. The correlations between the accumulation of these imidazoles and the defects in the histidine enzymes are consistent with the L-histidine biosynthetic sequence known in other microorganisms. Existence of both the aminotransferase and the dehydrogenase activities in the extracts from IG-producing mutants (KY-9080 and KY-9105) are also compatible with known histidine pathway.

Burke and Pattee[53] have reported that an extract from Staphylococcus aureus did not show the activities of HP phosphatase and HP aminotransferase. Two explanations were offered by these investigators for this observation. The

first was that HP is not an intermediate of the hisitidine pathway. A second and more favoured explanation was that the phosphatase and the aminotransferase were inhibited or inactivated during preparation of cell-free extracts or during assays for their activities.

In C. glutamicum studied here, HP phosphatase activity was not detected in the extracts from wild-type strains. Conversely, in the extracts from histidine auxotrophs grown with limited amount of L-histidine, weak but significant phosphatase activity was noted, though it is unclear whether the phosphatase activity is due to the phosphatase specific to HP or due to the nonspecific one which are differentiated each other in Saccharomyces cerevisiae.[54] As already described, HP aminotransferase activity could be determined in wild-type strains as well as in histidine auxotrophs of C. glutamicum, then the participation of HP in L-histidine biosynthesis in this microorganism appears to be likely. Accordingly, the failure to demonstrate the phosphatase activity in wild-type C. glutamicum strains is believed to be a technical one. It should be emphasized that the extracts employed here were prepared by sonicating the cells under the comparably drastic condition because of the rigidity of the cells.

Based on these considerations, it is assumed that at least the last four steps of the L-histidine biosynthetic sequence known in other microorganisms (Fig. 1 of Chapter I) are applicable to C. glutamicum.

SUMMARY

Two imidazoleglycerol (IG)-producing mutants and one imidazoleacetol(IA)-producing mutant were selected out of 14 histidine auxotrophs of C. glutamicum, by means of paper-chromatographic analysis of the culture broths of these mutants. Three of the L-histidine biosynthetic enzymes were determined for these mutants and a previously isolated L-histidinol-producing mutant of C. glutamicum. The IA-producing mutant and the L-histidinol-producing mutant had a defect in histidinol phosphate aminotransferase and histidinol dehydrogenase, respectively. IG-producers were not defective in these enzymes. These results were consistent with the L-histidine biosynthetic sequence known in other microorganisms.

Chapter III. Imidazoleglycerol Production with a Histidine Auxotroph of *Brevibacterium ammoniagenes*

INTRODUCTION

Imidazoleglycerol (IG) was found by Ames *et al.*[34,55] to be accumulated by histidine auxotrophs of *Neurospora* and *Penicillium*. This finding provided clues to the biosynthetic pathway of L-histidine in bacterial[29,39,40,53,56-59] and fungal[35,36] systems, in which the phosphate ester of it, *i.e.*, imidazoleglycerol phosphate serves as an intermediate but IG does not (see Fig. 1 in Chapter I).

During the course of the studies on amino acid production by microbial auxotrophs, the author found that a histidine auxotroph of *Brevibacterium ammoniagenes* produced a large amount of IG in the culture medium. The IG-productivity of this mutant was superior to that of the *Corynebacterium glutamicum* KY-9105 which was isolated as an IG-producer in the preceding chapter.

In the present chapter, the author describes the IG-production with the *B. ammoniagenes* mutant. The study aimed at L-histidine production from IG as a starting material.

MATERIALS AND METHODS

Microorganisms. A histidine auxotroph employed in this experiment was derived from a wild B. ammoniagenes strain, ATCC 6872, by the mutagenic treatment with UV-ray irradiation. The mutant strain has been deposited to The American Type Culture Collection with an accession number of ATCC 21225.

Culture method. Forty milliliters of a seed medium in a 250-ml Erlenmeyer flask was inoculated with a loopful of microorganism grown on a bouillon agar slant, and incubated at 28°C on a rotary shaker at 220 rpm. After incubation for 24 hr, one milliliter of the seed culture was transferred into the same type-flask containing 20 ml of a fermentation medium. This flask was incubated for 72 hr in the same manner as that with the seed culture. The seed medium contained glucose 4 g, peptone 2 g, meat extract 1 g, urea 0.3 g, KH_2PO_4 0.1 g, K_2HPO_4 0.1 g, $MgSO_4 \cdot 7H_2O$ 0.05 g, $ZnSO_4 \cdot 7H_2O$ 0.01 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, $MnSO_4 \cdot 4H_2O$ 0.0001 g, biotin 3 μg, thiamine·HCl 500 μg, β-alanine 500 μg per 100 ml. The fermentation medium contained $(NH_4)_2SO_4$ 2 g, urea 0.3 g, β-alanine 1.5 μg, $FeSO_4 \cdot 7H_2O$ 0.002 g, $ZnSO_4 \cdot 7H_2O$ 0.0001 g, $MnSO_4 \cdot 4H_2O$ 0.0001 g and $CaCO_3$ 3 g per 100 ml as basal ingredients. Glucose, natural nutrients, KH_2PO_4 and $MgSO_4 \cdot 7H_2O$ were added to the medium at variable levels according to the purpose of each experiment. The hydrogen ion concentration of the media was adjusted to pH 7.2 with NaOH, then the media were sterilized at 120°C for 10 min. The fermentation test shown in Fig. 5

was carried out in a 30-liters jar fermentor principally according to the method for the flask-test described above. The details of the test are described in the legend of Fig. 5.

Analysis. The determination of IG was based on its conversion to imidazoleformaldehyde by periodate oxidation. The assay was similar to that described by Ames et al.[60] for imidazoleglycerol phosphate, but has been scaled up to ten times the original scale. The standard curve was linear to IG concentration at least up to 40 μg of its monohydrochloride salt. In a sample containing 2.5 or 5.0 μg of IG (as monohydrochloride) added to a culture broth (containing 4.2 mg/ml of IG accumulated), the added IG was detected with an efficiency of 104 or 96%, respectively (Table I). A control containing an aliquot of a fermentation medium without being inoculated with microorganisms was assayed, and an appropriate correction was made using this control for the estimation of IG accumulated in the culture broth. The growth of microorganisms was measured by reading the absorbancy at 660 nm of the culture broth in a cuvette with a light-path of 0.5 cm using Tokyo Koden Medel ANA 7A Colorimeter, after dissolving the $CaCO_3$ in the medium with HCl and 20-fold dilution.

Table I. Determination of Imidazoleglycerol Added to a Culture Broth of B. ammoniagenes ATCC 21225

(A) Imidazoleglycerol monohydrochloride added (μg/ml)	(B) Imidazoleglycerol monohydrochloride determined (μg/ml)	Recovery ($\frac{B-4.2}{A}$ x 100,%)
0.0	4.2[a)]	
2.5	6.8	104
5.0	9.0	96

Samples containing indicated levels of imidazoleglycerol monohydrochloride in addition to 1 μl of a culture broth of ATCC 21225 per ml were assayed according to the method described in MATERIALS AND METHODS.

a) The value, 4.2 shows the amount of imidazoleglycerol accumulated in the culture broth.

Reagents. D-Erythro-imidazoleglycerol(IG) was kindly supplied by Dr. B. N. Ames. L-Histidinol was purchased from Sigma Chemical Company.

Isolation of IG. IG was isolated from the culture broth according to the procedure shown in Fig. 1.
It was done by passage the solution containing IG through the columns of H^+-form of cation exchange resins (Diaion SK#1 and Amberlite IRC-50) and by elution with N NH_4OH. The eluate was then concentrated to make 6.5% solution of IG, decolorized with carbon, adjusted to pH 3.0 with HCl, and further concentrated to dryness. IG was extracted from the residue with 98% ethanol and recrystallized in the form of monohydrochloride from the ethanol solution.

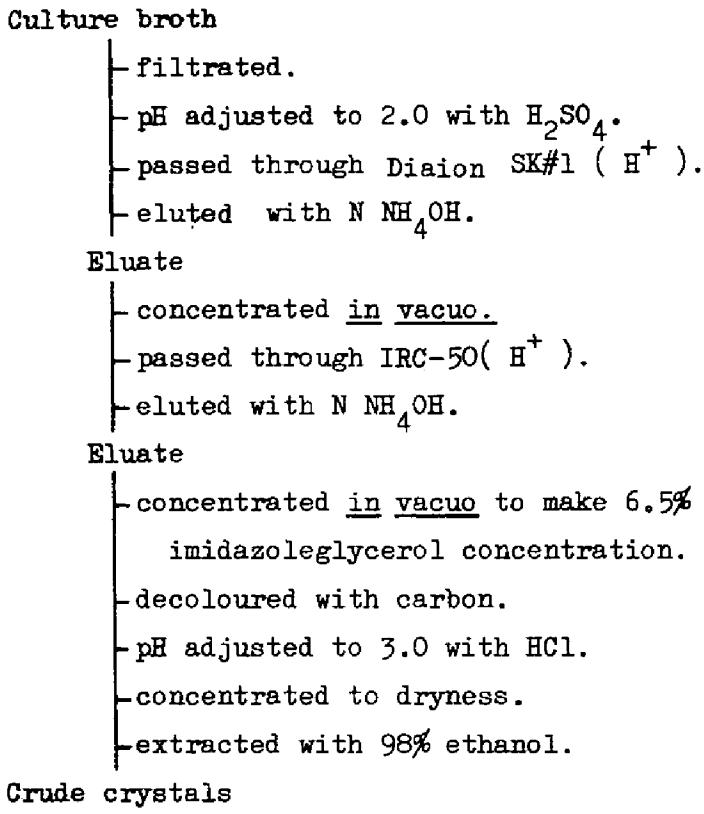

Fig. 1 Isolation of Imidazoleglycerol from Culture Broth

RESULTS

1. Identification of IG.

B. ammoniagenes ATCC 21225 was found to produce a diazosulfanilic acid reaction-positive substance in the culture broth. It was isolated in a crystalline form by the procedure described in MATERIALS AND METHODS section. $[\alpha]_D^{20}$ +10.18(C4, H_2O), m.p.

121.5 ~ 126.8°C.* Anal. Calcd. for $C_6H_{10}N_2 \cdot HCl$: C, 37.10 ; H, 5.67 ; N, 14.43. Found : C, 36.91 ; H, 6.34 ; N, 15.42. This compound had the same Rf-values with authentic IG in paperchromatography with five solvent systems, as shown in Table II. Like authentic IG, it was converted to imidazole-formaldehyde by oxidation with periodic acid, accomplished according to the method of Ames et al.[34] Moreover, it gave the same absorption spectrum of the chromatophore when reacted with diazosulfanilic acid[44] with that from authentic IG. The infrared spectrum of the crystal coincided with that of authentic IG as shown in Fig. 2. From these results, the substance produced by ATCC 21225 was concluded to be IG.

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* The melting point of the isolated crystal was in good agreement with that (126.5°C) of authentic IG, but it was somewhat higher than the value (102.5 ~ 103°C) described by Ames et al. in a previous paper.[34]

Table II. Paperchromatographic Identification of Imidazoleglycerol

Solvent system*	Imidazoleglycerol isolated from culture broth	Authentic imidazoleglycerol
a	0.565	0.565
b	0.476	0.476
c	0.156	0.156
d	0.096	0.106
e	0.409	0.409

*) Solvent system (ratio by volume): a, n-propanol-0.2N NH_4OH (3:1); b, n-propanol-N acetic acid (3:1); c, n-butanol-acetic acid-H_2O (5:2:2); d, acetone-chloroform-H_2O-28% NH_4OH (30:5:4:0.2); e, n-butanol-acetic acid-ethylacetate-H_2O (1:1:1:1).

Rf-values of the spots located with diazosulfanilic acid-reagent are given.

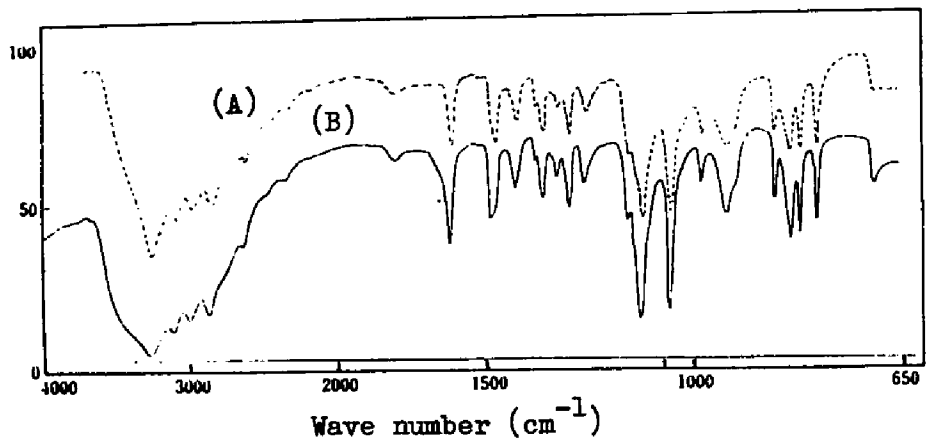


Fig. 2. Infrared Spectrum of Imidazoleglycerol (monohydrochloride).
A: Authentic imidazoleglycerol. B: Imidazoleglycerol isolated from broth.

2. Effect of growth factor.

In the fermentative production of amino acids with bacterial auxotrophs, supplying of the required substances in a level suboptimal for growth gives the maximal yield of the amino acids.[20] Because ATCC 21225 requires L-histidine for growth, the effect of L-histidine and natural nutrients containing L-histidine on IG-production was tested. As shown in Table III, more than 5 mg (as monohydrochloride) per ml of IG was produced when acid-hydrolyzate of C. glutamicum cells, acid-hydrolyzate of soybean meal or meat extract was added to the medium at 1~3% levels. At a high level (4%) of the natural nutrients, the yield of IG was distinctly reduced.

Table III. Effect of Natural Nutrients on Imidazoleglycerol Production by B. ammoniagenes ATCC 21225

Additions (%)		Growth(O.D.660X1/ 20)	Imidazoleglycerol accumulated*
Acid-hydrolyzate of C. glutamicum cells	(4)	0.78	3.2
	(3)	0.82	5.0
	(2)	0.84	6.8
	(1)	0.77	5.9
Acid-hydrolyzate of soybean meal	(4)	0.79	2.9
	(3)	0.78	6.8
	(2)	0.80	7.7
	(1)	0.66	5.5
Meat extract	(4)	0.62	1.3
	(3)	0.70	8.6
	(2)	0.59	8.1
	(1)	0.52	7.4
No addition		0.07	1.2

Fermentation medium basically contained 12% glucose, 0.2% KH_2PO_4, 0.2% Na_2HPO_4, 0.1% $MgSO_4 \cdot 7H_2O$ and the basal ingredients described in "MATERIALS AND METHODS."

*) mg/ml as monohydrochloride.

When L-histidine(monohydrochloride) was used as the growth factor, high amount (4.5 ~ 5.1 mg/ml, as monohydrochloride) of IG was produced under the condition with comparably high concentration, i.e., 600~1200 μg as monohydrochloride per ml or 3.1 ~ 6.2 mM of L-histidine(Fig. 3). Addition of much higher amount of L-histidine (2400 μg as monohydrochloride per ml) reduced the IG production only by 27% of the maximal yield. The cellular growth did not distincly respond to the L-histidine concentration upto 2400 μg (as monohydrochloride)/ml. These results are in remarked contrast with the case of L-histidinol production by a histidine auxotroph of C. glutamicum, KY-10234, where addition of 1 mM L-histidine to the medium gave the maximal yield of the product and increasing the L-histidine level to 2 mM reduced the yield by 50% or more (Chapter II). In this case, moreover, the growth of the mutant responded to the L-histidine concentration. The reason is unclear why the cellular growth and the IG production with B. ammoniagenes ATCC 21225 studied here were less effectively affected by L-histidine in comparison with the case of the L-histidinol production by the C. glutamicum mutant. Some difference between L-histidine permeabilities of these microorganisms, if it exists, might explain these phenomena.

At the L-histidine concentration of 75 ~ 300 μg as monohydrochloride per ml, the growth level of the IG producer was rather higher than the case with excess of L-histidine.

Such a high level growth under L-histidine-limitation has also been observed in the case of the above-described L-histidinol production by a histidine auxotroph of C. glutamicum. This may be ascribed to the appearance of non-auxotrophic revertants from ATCC 21225 during fermentation.

3. Effect of inorganic phosphate and magnesium.

Based on the informations on histidine pathway in other microorganisms,[29,35,36,39,40,53,56-59,61] it may be assumed that ATCC 21225 forms IG from its phosphate ester through the action of phosphatase. Because the phosphatase specific to imidazoleglycerol phosphate has not been found in microorganisms to our knowledge, it is possible that non-specific phosphatase may participate in its formation. Furthermore, inorganic phosphate is known to repress the formation of acid- and alkaline phosphatases in some microorganisms.[62,63] Accordingly, it was of interest to test the effect of inorganic phosphate on IG production by ATCC 21225. Effect of magnesium ion was also tested, because increasing of the concentrations of $MgSO_4 \cdot 7H_2O$ as well as KH_2PO_4 and K_2HPO_4 is shown[64] to stimulate the inosinic acid production by an adenine auxotroph of B. ammoniagenes. In the test, KH_2PO_4 and Na_2HPO_4, $MgSO_4 \cdot 7H_2O$ and meat extract were added to the medium as the sources of inorganic phosphate, magnesium ion and L-histidine, respectively. The results are shown in Table IV. It can be seen that high concentration (2%) of the phosphates reduced the growth when the concentration of the magnesium salt was low (0.05%). The growth-inhibition was released by the addition of high levels(1~2%) of the

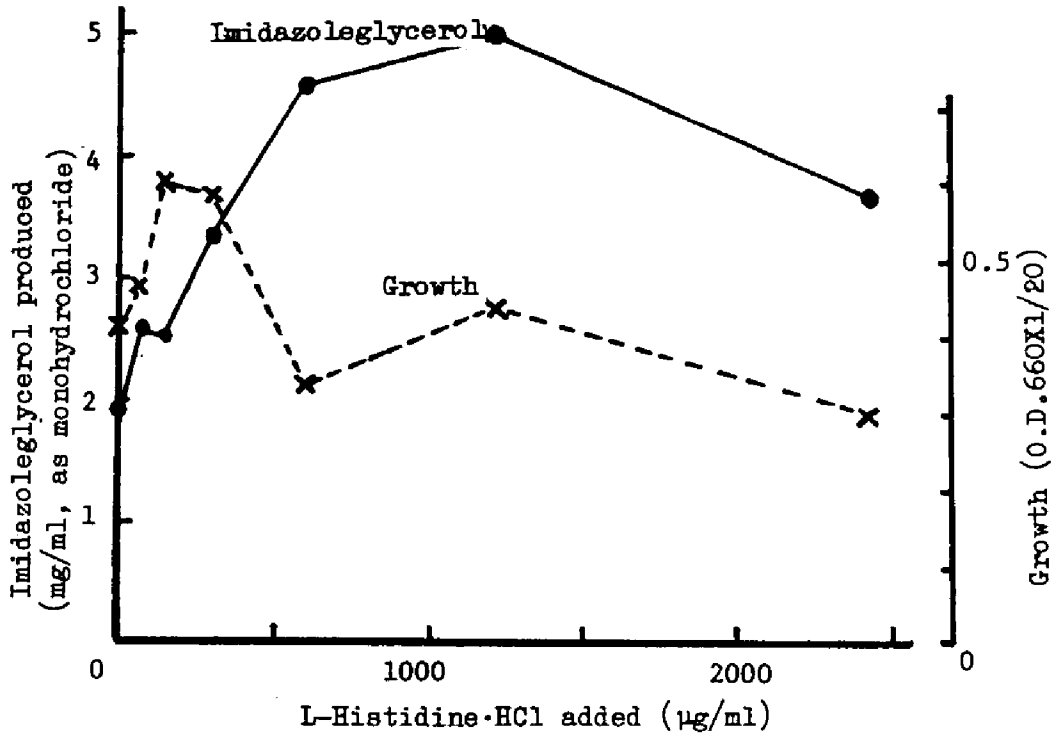


Fig. 3 Effect of L-Histidine on Imidazoleglycerol Production by B. ammoniagenes ATCC 21225

Fermentation medium was the same as that in Table III.

Table IV. Effect of Inorganic Phosphate and Magnesium Sulfate Concentration on Imidazoleglycerol Production

Phosphate salts added (%)	$MgSO_4 \cdot 7H_2O$ added (%)	Growth (OD660X1/20)	Imidazole-glycerol accumulated (mg/ml)*
KH_2PO_4(2) Na_2HPO_4(2)	2.00	0.63	13.1
	1.00	0.58	11.8
	0.05	0.46	10.7
KH_2PO_4(0.1) Na_2HPO_4(0.1)	2.00	0.68	11.6
	1.00	0.68	9.4
	0.05	0.61	12.1

Fermentation medium basically contained 12% glucose, 2.5% meat extract, and the basal ingredients described in MATERIALS AND METHODS.

* as monohydrochloride.

Phosphates added (%)	$MgSO_4 \cdot 7H_2O$ added (%)	Rf-value 0	0.5
KH_2PO_4(2) Na_2HPO_4(2)	2.00	±	++++ a)
	1.00	⊥	++++
	0.05	⊥	++++
KH_2PO_4(0.1) Na_2HPO_4(0.1)	2.00	⊥ +	++++ ⊥
	1.00	⊥ ⊥	++++ ⊥
	0.05	⊥ +	++++ +

Fig. 4. By-production of the Substances Positive to Diazosulfanilic Acid-reaction in Imidazoleglycerol Fermentation with B. ammoniagenes ATCC 21225.

The culture broth obtained in the experiment shown in Table IV was paperchromatogramed on a Toyo Roshi No.50 with a solvent system n-propanol-0.2N NH_4OH(3:1 by volume). The marks, ++++ , ++++ , + , ⊥ and ± show the locations of the coloured spots, in order of decreasing density.

a) Imidazoleglycerol.

magnesium salt to the medium. At the higher level (each 2%) of the phosphates, IG production increased with the increase of the growth levels or magnesium concentrations. At the low level (each 0.1%) of the phosphates, the concentration of magnesium ion did not affect the growth level. Moreover,

the correlation between magnesium ion concentration and IG production was obscure. The maximal amount of IG produced at the two different levels of the phosphates was almost the same, while by-production of other unidentified substances which were positive to diazosulfanilic acid-reaction was distinctly reduced at 2% level of the phosphates, as shown in Fig. 4.

4. Chemical changes during fermentation.

The time course of IG fermentation in a 30-liters jar fermentor is exemplified in Fig. 5. The production of IG paralleled with the growth until 30 hr and continued to increase even in the stationary phase of growth, accompanied by the consumption of glucose.

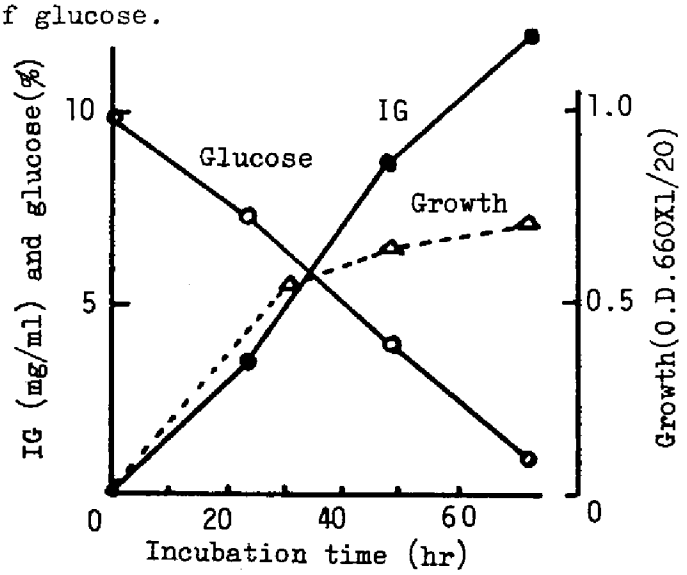


Fig. 5. Time Course of Imidazoleglycerol Fermentation

Fermentation was carried out in a 30-liters jar fermentor containing 15 liters of a fermentation medium comprising of 10% glucose, 3% meat extract, 2% KH_2PO_4, 2% Na_2HPO_4, 2% $MgSO_4 \cdot 7H_2O$ and basal ingredients.
Culture conditions: aeration, 10 liters/min; agitation, 400 rpm. The hydrogen ion concentration was automatically controlled with NH_4OH to pH 7.0 during fermentation.
IG : Imidazoleglycerol·HCl.

5. Trial of L-histidine production from IG

L-Histidine production from IG was attempted in the same manner as that described in the preceding chapter (Chapter II, Section 1) for the L-histidine production from L-histidinol. However, any significant amount of L-histidine was not detected in the culture broth.

DISCUSSION

A histidine auxotroph, B. ammoniagenes ATCC 21225 accumulated a large amount of IG in the culture medium. Though it has not been verified whether imidazoleglycerol phosphate is accumulated or not, its accumulation would be little, if it occurs, in view of the trace amount of other diazosulfanilic acid-positive spots on the paperchromatogram of the culture broth. These facts suggest that the histidine-auxotrophy of this mutant is due to the genetic block at imidazoleglycerol phosphate dehydratase (EC 4.2.1.19),[29,61] the enzyme converting imidazoleglycerol phosphate to imidazoleacetol phosphate in the histidine pathway. The reason for the preferential production of IG to its phosphate ester by this mutant may reside in the existence of the permeability barrier to the phosphate ester, the dephosphorylation of the phosphate ester through the action of some phosphatase, and excretion into the medium in the form of IG. This assumption is suggested by an interpretation relevant to permeability change, which has been made for the interchangeable accumulation of inosinic acid and hypoxathine by an adenine-auxotroph[65] derived from B. ammoniagenes ATCC 6872 from which ATCC 21225 studied here also originated.

SUMMARY

A histidine auxotroph of Brevibacterium ammoniagenes was found to accumulate imidazolegycerol in the culture medium. The accumulation of it reached a level of 13 mg as monohydrochloride per ml with a medium containing 12 % glucose, 2% $(NH_4)_2SO_4$ and 2.5 % meat extract. By-production of other imidazoles was little.

Chapter IV. L-Histidine Production with Histidine Analog-resistant Mutants of *Corynebacterium glutamicum*

Section 1. L-Histidine Production by Histidine Analog-resistant Mutants of Several Bacteria

INTRODUCTION

Excretion of L-histidine by histidine analog-resistant mutants has been known in *Salmonella typhimurium*,[66] *Escherichia coli*[67] and *Bacillus subtilis*.[68]

As described in Chapter II, a two-steps method for L-histidine production, i.e., production of L-histidinol with a histidine auxotroph of *Corynebacterium glutamicum*, and conversion of the L-histidinol into L-histidine has been developed. However, the yield of L-histidine was 4 mg/ml at highest.

The present investigation was undertaken to develope a direct process for L-histidine production, using a histidine analog-resistant mutant of bacteria.

MATERIALS AND METHODS

Microorganisms. Histidine analog-resistant mutants were derived from *C. glutamicum* ATCC 13761 (wild-type) and other bacteria which are listed in Table IX, by the mutagenic

treatment with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) according to the method of Adelberg et al.[69]

Culture method. Five milliliters of a seed medium (see Table I in Chapter II, Section 1) in a test tube (25 mm x 200 mm) were inoculated with one loopful of microorganisms grown on a bouillon agar slant, and incubated at 30°C on a test tube shaker. After incubation for 20 ~ 24 hr, the seed culture was transferred into a fermentation medium and incubated with the same manner as that described already (Chapter II, Section 2) for "imidazole accumulation". The basal compositions of the fermentation media as well as of a minimal medium used for the isolation of histidine analog-resistant mutants are shown in Table I. Sensitivity of the microorganisms to histidine-analogs was tested as follows: a test tube (16 mm x 160 mm) containing 2 ml of the minimal medium was inoculated with one drop of cell-suspension prepared from a bouillon agar slant to make the final cell concentration of 10^7 cells/ml, and incubated statically at 30°C for 24 hr.

Analysis. L-Histidine was colorimetrically determined according to the method of Mcpherson,[44] using the sample diluted with water. The growth in the medium was measured by reading the absorbancy at 660 nm of the culture in a cuvette with 10-mm light path, using a Hitachi Colorimeter Model 101. For the measurement of the growth in the fermentation medium, it was done after dissolving the $CaCO_3$ in the medium with HCl and 100-fold dilution and dry cell weight (D.C.W.) was calculated from the absorbancy by means of an appropriate calibration curve.

Table I. Basal Composition of Media

Ingredients (amount in 100 ml)	MM*	G*	M*
Glucose (g)	1	15	
Cane molasses (g)**			15
$(NH_4)_2SO_4$ (g)	0.4	2	2 or 4.5
Urea (g)	0.1	0.3	0.3
KH_2PO_4 (g)		0.15	0.15
K_2HPO_4 (g)	0.01	0.05	0.05
$MgSO_4 \cdot 7H_2O$ (g)	0.05	0.05	0.05
$FeSO_4 \cdot 7H_2O$ (g)		0.01	
$MnSO_4 \cdot 4H_2O$ (g)		0.01	
$CaCO_3$ (g)		3	3
Biotin (μg)	6	20	
Thiamine·HCl (μg)	500	30	
Trace element soln.***(ml)	0.1		

*) MM: Minimal medium. G: Glucose medium.
M: Molasses medium.

**) As glucose.
The hydrogen ion concentration of the media was adjusted to pH 7.4 with NH_4OH, and the media were sterlized at 120°C for 10 min.

***) See Ref. 126).

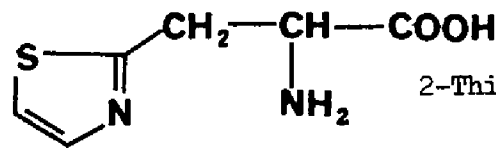
Reagents. 1,2,4-Triazole-3-alanine (TRA) and 2-thiazolealanine (TA) were purchased from Eli Lilly & Co. L-β-Imidazolelactic acid was purchased from Sigma Chemical Co.

RESULTS

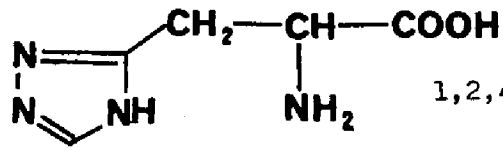
1. Derivation of histidine analog-resistant mutants from C. glutamicum ATCC 13761

Attempts were made to derive the mutant strains resistant to histidine-analogs, 1,2,4-triazolealanine (TRA) and 2-thiazolealanine (TA), from a wild-type C. glutamicum strain, ATCC 13761.

The chemical structures of the histidine-analogs are shown in Fig. 1.



2-Thiazolealanine (TA)



1,2,4-Triazole-3-alanine (TRA)

Fig. 1. Histidine Analogs

When the NTG-treated cells were spread on the minimal agar plate containing 1000 μg/ml of TRA or 750 μg/ml of TA at the cell concentration of 10^5 or 10^6 per plate, the resistant colonies appeared on the plate as shown in Table II. The frequency of the appearance of the resistant colonies was approximately 10^{-3} or 10^{-4} with TA or TRA, respectively.

Table II. Derivation of Histidine Analog-resistant Mutants from C. glutamicum ATCC 13761

No. of cells spread per plate	Analog added (μg/ml)	Colony count per plate	
		Large colonies	Small colonies
10^6	TRA (1000)	120	> 890
	No addition	Dense growth*	
10^5	TRA (1000)	32	80
	TA (750)	240	360
	No addition	Dense growth*	

*) Dense growth on the whole surface of the agar plate. NTG-treated cells of ATCC 13761 were spread on the MM-agar medium containing the indicated levels of histidine-analogs. After incubation for 3 days, colonies were counted.

2. Resistance to histidine-analogs.

The resistance to TRA and TA of a mutant strain TRA-57, which was derived on TRA-agar, was compared with that of the parent wild-type strain ATCC 13761. It was done in a minimal liquid medium containing TRA and TA at various concentrations according to the method described in "MATERIALS AND METHODS." As shown in Fig. 2, the growth of the parent strain decreased by 60 % and 70 % by the addition of 50 μg/ml of TRA and TA, respectively. On the other hand, the strain TRA-57 exhibited the resistance to the inhibition of both histidine-analogs in comparison with the parent strain. The TRA-resistant nature of TRA-57 also confirmed by the growth test with the minimal agar medium. The mutant strain grew well on the TRA-agar

containing 300 μg/ml of TRA, while the parent strian did not. The similar results were obtained with KY-10260 which was derived as a TRA-resistant. These results indicate that some mutants derived as TRA-resistant, including TRA-57 and KY-10260, are cross-resistant to TRA and TA.

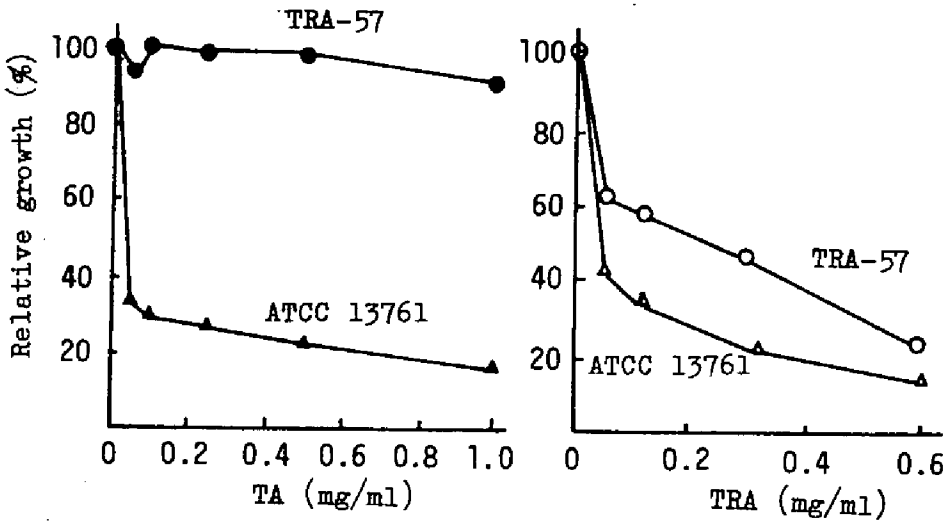


Fig. 2 Resistance of Mutant TRA-57 to Histidine-analogs.

Relative growth (%) is given, taking the turbidity of each strain in a non-supplemented medium as 100 %.

3. L-Histidine production by histidine analog-resistant mutants.

L-Histidine production by histidine analog-resistant mutants from C. glutamicum ATCC 13761 was investigated.

As shown in Table III, 93 and 86% of the mutants derived on each of the TRA-agar and the TA-agar produced more than 0.05 mg/ml of L-histidine in the culture medium. Among the L-histidine producers, a mutant strain KY-10260 which was derived as a TRA-resistant was investigated for the culture conditions for L-histidine production. L-Histidine was isolated from the culture broth of this mutant and identified from its physical and chemical properties.

Table III. L-Histidine Production by Histidine Analog-resistant Mutants from *C. glutamicum* ATCC 13761

Resistant to	No. of strains tested	No. of strains producing L-histidine in amount of		
		0.05~1.0 (mg/ml)	1.0~2.0 (mg/ml)	2.0~3.4 (mg/ml)
TRA	113	72	21	13
TA	72	45	7	10

Fermentation medium: M-medium with 2% $(NH_4)_2SO_4$.
Incubation time: 3 days.

4. Chemical changes during fermentation.

Fig. 3 exemplifies the chemical changes during the fermentation in a cane molasses medium and a glucose medium. In each medium, L-histidine production paralleled with the growth of the microorganism and reached maximum in 2 to 3 days. L-Histidine concentration did not decline regardless of a rapid raise of pH-value on 4th day.

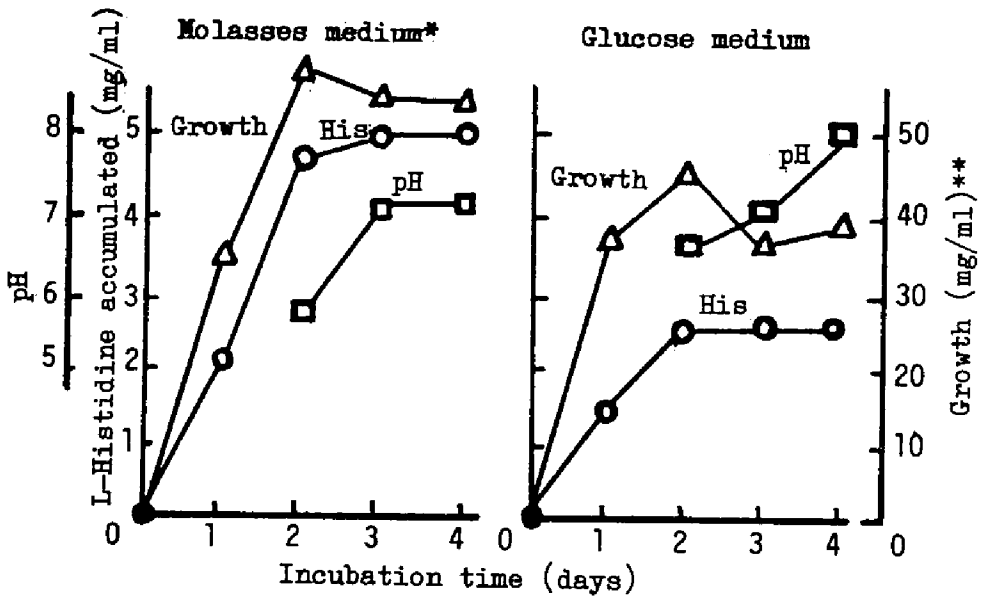


Fig. 3 Time Course of Histidine Fermentation with C. glutamicum KY-10260

* With 2% $(NH_4)_2SO_4$ ** Dry cell weight

5. Effect of carbon source on L-histidine production.

Six sugars and cane molasses were tested at 10 % level to determine the favourable carbon source for L-histidine production. As shown in Table IV, fructose, maltose, mannitol, mannose, sucrose and glucose gave comparable yields. With molasses, the L-histidine production was twice as much as with other carbon sources.

Table IV. Effect of Carbon Sources on L-Histidine Production by C. glutamicum KY-10260

Sugar (10 %)	L-Histidine produced (mg/ml)
Fructose	2.5
Maltose	2.4
Mannitol	2.5
Mannose	2.7
Sucrose	2.9
Glucose	2.4
Molasses	5.1

G-medium (missing glucose) was used as the basal medium. Incubation time: 3 days.

6. Effect of nitrogen source on L-histidine production.

Five nitrogen sources were investigated at various concentrations to determine the favourable nitrogen source and its concentration for L-histidine production in a cane molasses medium. Except for urea, every nitrogen source gave good yield at the concentrations higher than 2%. With 4 to 5% $(NH_4)_2SO_4$ or 3% NH_4NO_3, L-histidine accumulation was maximum and reached a level of 6 mg/ml (Fig. 4).

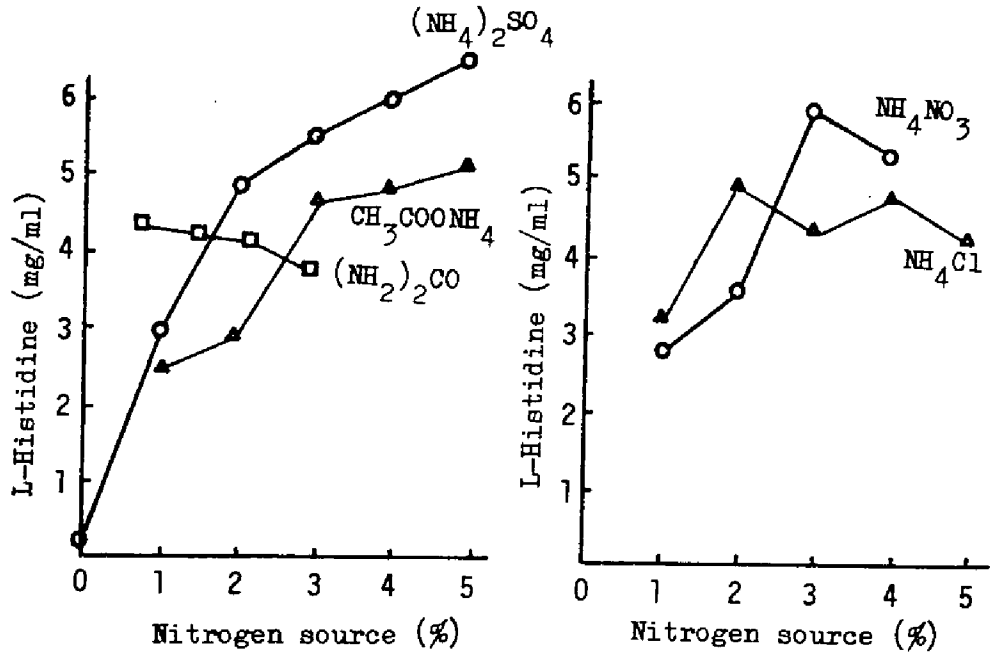


Fig. 4. Effect of Nitrogen Source on L-Histidine Production by C. glutamicum KY-10260

M-medium [missing $(NH_4)_2SO_4$ and urea] was used as the basal mediu
Incubation time: 4 days.

7. Effect of various substances on L-histidine production.

Various substances were tested for their effect on the L-histidine production by KY-10260. It was done by the addition of following substances into the cane molasses medium: casamino acids, peptone, meat extract, yeast extract, corn steep liquor, NZ-amine, acid-hydrolyzate of soybean meal, acid-hydrolyzate of C. glutamicum cells (at the levels of 0.25, 0.5 and 1.0 %); 22 amino acids (100 and 1000 μg/ml); 4 purines (20 and 1000 μg/ml); 3 pyrimidines (20 and 1000 μg/ml), 9 vitamins (10 and 1000 μg/ml);

organic acids such as oxalic acid, gluconic acid, lactic acid, acetic acid, pyruvic acid, citric acid, succinic acid, tartaric acid, malic acid, formic acid, fumaric acid, α-ketoglutaric acid and itaconic acid (0.5 and 1.0 %). The substances which were found to affect the L-histidine production are shown in Table V. Any of these supplements did not stimulate the L-histidine production so extensively as expected. The maximal yield (8 mg/ml) was obtained by the addition of 1 % acetic acid. L-Glutamine and L-tryptophan lowered the L-histidine production.

Table V. Effect of Various Substances on L-Histidine Production by C. glutamicum KY-10260

Substances added		L-Histidine produced (mg/ml)
Corn steep liquor	(1.0 %)	6.7
	(0.5 %)	6.8
NZ-amine	(1.0 %)	7.0
Meat extract	(1.0 %)	6.8
	(0.5 %)	6.9
Peptone	(0.75%)	6.9
No addition		6.0
L-Glutamine	(1000 μg/ml)	4.5
L-Tryptophan	(200 μg/ml)	4.5
No addition		6.1
Acetic acid	(1.0 %)	8.0
Pyruvic acid	(0.5 %)	7.3
No addition		6.5

M-medium with 4.5 % $(NH_4)_2SO_4$ was used as the basal medium.
Incubation time: 3 days

8. Effect of L-histidine on L-histidine production.

As described above, L-histidine accumulated at the concentration of 6-8 mg/ml in the culture medium of KY-10260. To determine whether such a high concentration of L-histidine in the medium inhibits the production of L-histidine itself or not, the L-histidine producer was cultured in the glucose medium supplemented with 5 and 10 mg/ml of L-histidine. As shown in Table VI, approximately the same amount of L-histidine was produced with or without addition of L-histidine. This result indicates that L-histidine does not inhibit the L-histidine production by the mutant at least at the level of 10 mg/ml.

Table VI. Effect of L-Histidine on L-Histidine Production by C. glutamicum KY-10260

L-Histidine added (mg/ml)	Growth (mg/ml as D.C.W)	L-Histidine (mg/ml)	
		Total	Produced
10.0	45	12.4	2.4
5.0	40	7.0	2.0
0.0	37	2.7	2.7

Basal medium: G-medium. Incubation time: 3 days.

9. By-products.

As shown in Fig. 5, β-imidazolelactic acid and glycine were detected on a paperchromatogram of a culture broth of L-histidine producers each by the diazosulfanilic acid-reaction

and ninhydrin-reaction as by-products. Identification of these by-products was obtained in comparison with authentic samples in paperchromatography as shown in Table VII and VIII. The amount of glycine was usually 2~4 mg/ml, and that of imidazolelactic acid varied depending on the strain used. For example, the amount of the imidazolelactic acid produced by KY-10260 in the molasses medium was less than 400 μg/ml, while it reached the level of 500 ~ 2000 μg/ml by another L-histidine producer KY-10261 which was derived as a TA-resistant.

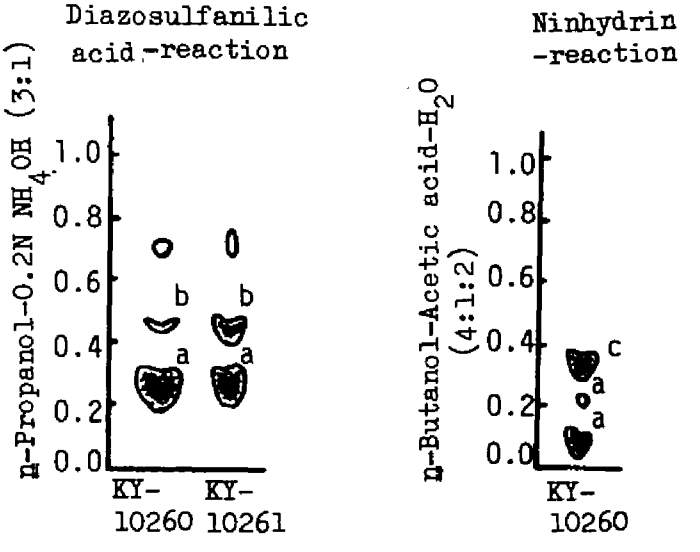


Fig. 5 By-products of Histidine Fermentation
a) L-Histidine. b) Imidazolelactate. C) Glycine.

Table VII. Identification of β-Imidazolelactic Acid

Diazosulfanilic acid-reaction (Rf)

Solvent system \ Sample	Sample from culture broth (KY-10261)	L-β-Imidazole lactic acid
a	0.37	0.37
b	0.31	0.31
c	0.07	0.07
d	0.46	0.46
e	0.57	0.58
f	0.63	0.63

a: n-Butanol-Acetic acid-H_2O(4:1:2); b:n-Propanol -0.2N Ammonia(3:1); c:N Ammonia-saturated n-butanol; d:t-Butanol-H_2O-Methylethylketone-Diethylamine (80:30: 40:8); e:Ethanol-H_2O(77:23); f:t-Butanol-Formic acid-H_2O(70:15:15)

Table VIII. Identification of Glycine (Rf)

Ninhydrin-reaction

Solvent system \ Sample	Sample from culture broth (KY-10260)	Glycine
a	0.17	0.17
d	0.34	0.34
g	0.10	0.10
h	0.40	0.40
o-Phtalaldehyde-reaction		
a	0.16*	0.16*
d	0.38*	0.37*

g: Methylethylketone-n-Butanol-H_2O-28% Ammonia(100: 100:60:5); h:Water-saturated phenol.

*Green spot

10. L-Histidine production by histidine analog-resistant mutants derived from other bacteria.

Derivation of histidine analog-resistant mutants from C. glutamicum ATCC 13761 and their L-histidine production were described above.

The similar procedure was applied to another C. glutamicum strain and other genera of bacteria, including Arthrobacter, Brevibacterium, Bacillus and Nocardia. As shown in Table IX, L-histidine producer could be obtained from them.

Table IX. L-Histidine Production by Histidine Analog-resistant Mutants from Several Bacteria

Parent strain (ATCC No.)	Resistant to (μg/ml)	No.of strains tested	Media	No. of strains producing L-histidine in amount of			
				<0.05 mg/ml	0.05 ~1.0 mg/ml	1.0 ~2.0 mg/ml	2.0 ~3.4 mg/ml
A.citreus (11624)	TA (1000)	31	G	29	2	0	0
B.flavum (14067)	TA (1000)	31	G	24	7	0	0
			M*	25	0	2	4
B.megaterium (10778)	TA (250)	20	G	17	3	0	0
B.subtilis (15244)	TRA (125)	13	G	11	2	0	0
C.glutamicum (13032)	TA (1000)	25	M*	18	6	0	1
N.globerula (13130)	TA (1000)	21	G	20	1	0	0

*) M-Medium with 2% $(NH_4)_2SO_4$.

DISCUSSION

It was now been demonstrated that histidine analog (TA or TRA)-resistant mutants derived from several bacteria produced a considerable amount of L-histidine in the culture medium.

In S. typhimurium, it is known that TRA causes repression of the histidine operon[71] and TA inhibits phosphoribosyl-ATP pyrophosphorylase (EC 4.1.2c)[60] which is the first enzyme of the histidine pathway. Some mutants resistant to TRA have the derepressed levels of the L-histidine biosynthetic enzymes[70] and the mutants resistant to TA have the first enzyme which is resistant to the feedback inhibition of L-histidine.[66] Recently, Fink et al.[45] reported that a TRA-resistant mutant of Saccharomyces cerevisiae had the pyrophosphorylase which is resistant to the feedback inhibition. The L-histidine biosynthetic pathway of the L-histidine producers studied here is believed to be insensitive to the metabolic regulation like that of the S. typhimurium mutants and the S. cerevisiae mutant.

At least two mutants of C. glutamicum, TRA-57 and KY-10260, which were derived as TRA-resistants exhibited resistance to TA as well as to TRA, and the extent of the resistance to TA was greater than that to TRA. It needs a further work to clarify the nature of the change of regulation mechanism in the histidine analog-resistant mutants derived in the present study.

Some culture conditions for L-histidine production by a selected L-histidine producer, C. glutamicum KY-10260, were investigated. Production of L-histidine was better in the

molasses medium than in the glucose medium. Cane molasses seems to contain some factors which are favourable to the L-histidine production.

β-Imidazolelactic acid was identified as a by-product of the histidine fermentation. A minute amount of this imidazole was also formed in the L-histidine-supplemented culture of the wild-type strain ATCC 13761. Therefore, the imidazole must be formed through the metabolism of L-histidine, presumably *via* imidazolepyruvic acid as shown in *E. coli*[72] and *Proteus vulgaris*.[73] The scarcity of the L-histidine-degradation would be another reason of the L-histidine accumulation by the *C. glutamicum* mutants.

SUMMARY

The mutant strains resistant to 1,2,4-triazolealanine (TRA) or 2-thiazolealanine (TA) were derived from *Corynebacterium glutamicum* ATCC 13761 by the mutagenic treatment with N-methyl-N'-nitro-N-nitrosoguanidine. More than eighty percent of these mutants were found to accumulate L-histidine in the culture medium. Among these L-histidine producers, a strain KY-10260 which was derived on TRA-containing medium, was used to investigate the culture conditions for L-histidine production. The amount of L-histidine accumulated reached a level of 6~8 mg/ml with a medium containing 15 % (as glucose concentration) cane molasses and 4.5 % $(NH_4)_2SO_4$.

According to the similar procedure, some L-histidine producers were derived from other bacteria.

Section 2. L-Histidine Production by Auxotrophic Histidine Analog-resistant Mutants of *Corynebacterium glutamicum*

INTRODUCTION

As described in the preceding section, histidine analog-resistant mutants of several bacteria produced a large amount of L-histidine in the culture medium. A selected mutant strain *Corynebacterium glutamicum* KY-10260 which was derived as 1,2,4-triazole-3-alanine (TRA)-resistant produced 6~8 mg/ml of L-histidine. The production of L-histidine is explained in terms of the lack of the feedback regulation on the histidine pathway by the end product, L-histidine.

Some reports implicated L-histidine in the regulation mechanism of aromatic amino acid biosynthesis of *Bacillus subtilis*[74,75] and *Neurospora crassa*.[76] The regulatory relationship between L-histidine and the synthesis of aromatic amino acids in *B. subtilis* exemplifies "metabolic interlock" termed by Jensen.[77] A reverse relationship, *i.e.*, the regulation of histidine pathway by aromatic amino acid is also known in *B. subtilis*.[78] Based on these informations, a high production of L-histidine is expected to be given by the auxotrophic mutants of *C. glutamicum* carrying some defects in addition to the histidine analog-resistance, though it is not known whether any other metabolite than L-histidine

participates in the regulation of the histidine pathway of this microorganism or not.

In the present investigation, the author derived mutants of C. glutamicum which have these two markers and tried to select a useful mutant strain for L-histidine production from them.

MATERIALS AND METHODS

Microorganisms. C. glutamicum mutants carrying auxotrophy in addition to histidine analog-resistance were derived by mutagenic treatment with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) in the following two ways. One was the derivation of 2-thiazolealanine (TA)-resistant mutants from a tyrosine auxotroph KY-9296, a phenylalanine auxotroph KY-9182, and a tryptophan auxotroph KY-9292 all of which were derivatives of a wild-type strain C. glutamicum ATCC 13032. The other was the derivation of a wide variety of auxotrophic mutants from a TRA-resistant L-histidine producer, KY-10260, a derivative of another wild-type strain, C. glutamicum ATCC 13761.

Culture method. Fermentation for L-histidine production was carried out in a 250-ml Erlenmeyer flask for 72 hr by the manner described in the preceding section, unless otherwise stated. The compositions of the fermentation medium are shown in the legend of each table and figure. The fermentation test shown in Table VI was carried out with a 5-liters jar fermentor principally according to the flask test. The

details of the experiment are shown in the legend of the table.

Determination of nutritional requirement. The cells of auxotrophic mutants were streaked on minimal agar medium supplemented with a mixture of vitamins, base components of nucleic acids or amino acids. From the growth behavior in three-days incubation at 30°C on the media, they were classified into the five groups shown in Table IV. The above-described supplements were used to make final concentrations as follows : 1) Vitamins - choline 1000μg, folic acid 50 μg, inositol 500 μg, calcium pantothenate 50 μg, pyridoxine hydrochloride 50 μg, riboflavin 250 μg, each per liter. 2) Base components of nucleic acids - adenine, guanine, hypoxanthine, xanthine, cytosine, thymine, uracil, each 5 μg/ml. 3) Amino acids - arginine, asparagine, aspartic acid, alanine, citrulline, cysteine, cystine, glutamic acid, glutamine, histidine, homoserine, isoleucine, leucine, lysine, methionine, ornithine, proline, phenylalanine, serine, threonine, tryptophan, tyrosine, valine, each 20 μg/ml with respect to L-isomer.

Analysis. L-Histidine and the growth in the medium were measured by the method described in the previous chapter (Chapter II, Section 1). L-Valine was measured by the ninhydrin method after paperchromatographic separation.

RESULTS

1. L-Histidine production by TA-resistant derivatives of aromatic amino acid auxotrophs.

As described previously (Section 1 of this chapter), a histidine analog-resistant mutant of C. glutamicum accumulated 6~7 mg/ml [4.0~4.7 % (w/w) of the initial sugar] in the culture medium containing 15 % sugar.

Following informations led us to expect possible improvement of the L-histidine producer by selecting aromatic amino acid-auxotrophic mutant: Nester et al.[79] have reported that L-tyrosine regulates the L-histidine biosynthesis in B. subtilis. This includes the growth inhibition of a histidine bradytroph by L-tyrosine, its reversion by L-histidine, L-tyrosine repression of histidine gene-products,[78,80] and L-tyrosine inhibition of phosphoribosyl-ATP pyrophosphorylase,[80] the first enzyme of L-histidine biosynthesis. Though there is no evidence for the regulation of L-histidine biosynthesis by L-phenylalanine or L-trytophan, to our knowledge, the regulation of the biosynthesis of these aromatic amino acids by L-histidine has also been known in B. subtilis[75,78,81] and Neurospora crassa.[76,82]

The author isolated TA-resistant mutants from aromatic amino acid-auxotrophs, C. glutamicum KY 9296 (tyr), KY 9182 (phe) and KY-9292 (try), on a minimal agar medium supplemented with 1000 μg/ml of TA in addition to 100 μg/ml of an aromatic amino acid required by each parent strain. The L-histidine production by them was investigated in a medium containing 10% (as glucose concentration) cane molasses and

1% yeast extract. The yeast extract was used as the source of aromatic amino acids required by the mutants. Table I shows that more than 60% of TA-resistant mutants from three parent strains accumulated more than 0.5 mg/ml of L-histidine in the culture medium. The level of L-histidine produced was, however, 2~3.4 mg/ml or 2~3.4%(w/w) of the initial sugar, at best. The L-histidine production and the growth of three representative strains which were selected in this experiment are given in Table II. As can be seen, a TA-resistant

Table I. L-Histidine Production by Thiazolealanine-resistants Derived from Aromatic Amino Acid Auxotrophs of C. glutamicum.

Parent strain (requirement)	No. of TA-resistants tested	No. of strains which produced L-histidine in amount (mg/ml) of		
		0.5~1.0	1.0~2.0	2.0~3.4
KY-9296 (tyr)	48	4	11	21
KY-9182 (phe)	47	2	8	20
KY-9292 (try)	47	10	19	3

Fermentation medium: Molsses 10% (as glucose concentration), $(NH_4)_2SO_4$ 4.5%, yeast extract 1%, urea 0.3%, KH_2PO_4 0.15%, K_2HPO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCO_3$ 3%.

Table II. Comparison of Representative L-Histidine Producers Selected from Thiazolealanine-resistants Derived from Aromatic Auxotrophs with KY-10260.

L-Histidine producer (phenotype)	L-Histidine produced (mg/ml)	Growth (O.D.660 x 1/100)
TYT-6 (tyr,TA^r)	3.4	0.22
PET-35 (phe,TA^r)	3.3	0.55
TRYT-17 (try,TA^r)	2.2	0.22
KY-10260 (TRA^r)	4.2	0.60

The representative strains were selected in the experiment shown in Table I.

tyrosine auxotroph TYT-6 produced L-histidine comparably with a TRA-resistant mutant KY-10260 which had been derived from a wild-type strain, in spite of the lower growth level. TYT-6 was further tested using fermentation media supplemented with other natural nutrients. As shown in Table III, the growth of TYT-6 was significantly stimulated by the addition of 2~3% NZ-amine (an enzymatic digest of casein, a product of Sheffield Co., U. S.), 3% enzyme-hydrolyzate of sake cake, 2% peptone, 1-3% "Mieki" (a kind of protein hydrolyzate), or 2~3% meat extract in comparison with the case when 1% yeast extract was added. The level of L-histidine production was 6.0 mg/ml or 4% (w/w) of the initial

sugar. These values were the same with or less than those obtained with KY-10260.

Table III. Effect of Natural Nutrients on L-Histidine Production by a Thiazolealanine-resistant Tyrosine Auxotroph of *C. glutamicum*.

Natural nutrient added(%)		L-Histidine produced (mg/ml)	Growth (O.D.660 x 1/100)
Corn steep liquor	(3)	6.0	0.44
	(2)	3.5	0.40
	(1)	4.4	0.35
NZ-amine	(3)	5.1	0.58
	(2)	5.0	0.51
	(1)	4.1	0.46
Enzyme-hydrolyzate of sake cake	(3)	5.1	0.56
	(2)	4.5	0.41
	(1)	4.5	0.37
Peptone	(3)	4.8	0.47
	(2)	4.8	0.51
	(1)	4.1	0.38
"Mieki"	(3)	2.6	0.52
	(2)	2.8	0.62
	(1)	5.0	0.53
Meat extract	(3)	4.4	0.56
	(2)	3.4	0.55
Yeast extract	(3)	4.1	0.44
	(2)	3.7	0.40
	(1)	3.8	0.38

Basal composition of the fermentation medium used was the same as that in the footnote of Table I except that molasses concentration was increased to 15% (as glucose concentration) and yeast extract was omitted.
Strain: TYT-6.

2. L-Histidine production by auxotrophic derivatives of KY-10260

One hundred and sixty-four auxotrophic mutants were derived from a TRA-resistant L-histidine producer, C. glutamicum KY-10260. Their L-histidine productivity was tested using a medium containing 15% (as glucose concentration) molasses and 1% yeast extract. Distribution of these mutants with respect to their L-histidine production is summarized in Table IV, together with the nutritional requirements. As can be seen, 28 out of 164 auxotrophs tested produced L-histidine in higher amount compared with the parent strain KY-10260. These 28 strains were repeatedly cultured by the same manner. Table V shows the results of two experiments on six representative strains. An auxotroph, Rα -88 repeatedly gave distinctly higher production of L-histidine compared with the parent strain and the level of production reached 7.3 ~ 8.7 mg/ml or 4.9 ~ 5.8% (w/w) of the initial sugar. The higher production of L-histidine by Rα -88 compared with KY-10260 was confirmed also by the fermentation with a 5-liters jar fermentor (Table VI). Though other mutants, Rα -29, Rα -30, Rα -48 and Rα -119, produced a comparably high level of L-histidine in the experiments shown in Table V, their obvious superiority over the parent strain was not reproduced in a jar fermentor-test. Rα -88 was proved to be a leucine auxotroph.

3. Effect of molasses-sucrose ratio on L-histidine production.

Molasses was previously shown to be a more favourable carbon source than glucose or sucrose for L-histidine production

Table IV. L-Histidine Production by Auxotrophic Derivatives of C. glutamicum KY-10260

Group of mutants*	No of strains tested	No. of strains which produced L-histidine in amount (%)** of		
		<80	80~100	100~160
A	53	27	14	12
B	36	31	4	1
V	15	8	1	6
X	32	23	4	5
W	28	15	9	4

Fermentation medium: The same as that used in the experiment shown in Table I, except for the increase of molasses concentration to 15% (as glucose concentration).

*) Mutants indicated with mark A, B or V are auxotrophs (or bradytrophs) whose growth responded to a mixture of amino acids, base components of nucleic acids or vitamins, respectively. Mutants with mark X are auxotrophs which did not respond to any of the amino acids, bases and vitamins. Mutants with mark W are bradytrophs whose requiring substances have not been identified.

**) The amount of L-histidine accumulated by the parent strain KY-10260 was taken as 100%. It ranged between 5.4 ~ 6.0 mg/ml in three repeated experiments.

by KY-10260. Moreover, it most remarkably stimulated the L-histidine production among various substances tested (Section 1 of this chapter). This suggests that molasses contains some

Table V. Representative L-Histidine Producers Selected from Auxotrophic Derivatives of a Triazolealanine-resistant L-Histidine Producer, C. glutamicum KY-10260.

Strain No.	Group of mutant	L-Histidine produced (mg/ml)		Growth (O.D.660 x 1/100)	
		Exp.I	Exp.II	Exp.I	Exp.II
Rα -29	W	6.8	6.1	0.64	0.66
Rα -30	B	6.0	5.9	0.66	0.70
Rα -48	A	7.5	6.7	0.68	0.70
Rα -79	A	6.3	5.8	0.64	0.70
Rα -88	A(leu)	8.7	7.3	0.66	0.51
Rα -119	A	6.3	7.3	0.66	0.59
KY-10260	(Parent)	5.4~6.0	5.7	0.66~0.70	0.70

Fermentation medium: the same as that shown in the footnote of Table IV.

Table VI. L-Histidine Production by Representative L-Histidine Producers Selected from Auxotrophic Derivatives of C. glutamicum KY-10260

Strain No.	L-Histidine produced (mg/ml)	Growth (mg/ml)*
Rα -29	3.8	29
Rα -30	5.8	38
Rα -48	4.0	36
Rα -88	8.1	30
Rα -119	5.7	40
KY-10260 (Parent)	4.2	41

Fermentation was carried out in a 5-liters jar fermentor containing 3 liters of a medium comprising of 15% (as glucose concentration) molasses, 0.2% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 2% $(NH_4)_2SO_4$, 0.2% urea, and 0.5% yeast extract.

Culture condition: agitation, 600 rpm; aeration, 5 liters/min; temperature, 30°C; incubation time, 76 hr. Hydrogen ion concentration of the culture was adjusted to pH 7.0 during fermentation with NH_4OH.

*) Dry cell weight.

Table VII. Effect of Molasses-Sucrose Ratio on L-Histidine Production by a *C. glutamicum* Mutant Rα -88.

Strain No.	Percentage of molasses[a)]	L-Histidine produced (mg/ml)	Growth (O.D.660 x 1/100)
Rα -88	0.0	4.1	0.42
	37.5	6.5	0.57
	50.0	9.5	0.60
	62.5	11.0	0.57
	100.0	8.5	0.70
KY-10260	0.0	3.0	0.46
	37.5	9.2	0.60
	50.0	8.6	0.64
	62.5	8.1	0.69
	100.0	6.8	0.70

Fermentation medium: Sugar 20%, KH_2PO_4 0.2%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, urea 0.2%, $(NH_4)_2SO_4$ 4%, $FeSO_4 \cdot 7H_2O$ 0.001%, $MnSO_4 \cdot 4H_2O$ 0.001%, corn steep liquor 0.5%, biotin 200 µg/L, thiamine·HCl 250 µg/L, $CaCO_3$ 3%.

a) Both of molasses and sucrose were used as source of sugar, singly or in combination. The percent sugar concentration of molasses to a total sugar used is given.

substances effective on the L-histidine production. Therefore, the effect of molasses concentration on L-histidine production by Rα-88 selected in the preceding experiment was tested. It was done employing the media which contained 20% sugar consisting of molasses and sucrose in different ratios. As shown in Table VII, the level of L-histidine production by the mutant reached 11 mg/ml or 5.5%(w/w) of the initial sugar at the optimal concentration of molasses. The level of L-histidine production by KY-10260 was 9.2 mg/ml or 4.6% of the initial sugar.

4. Effect of L-leucine on L-histidine production

Leucine auxotrophs of C. glutamicum have been reported to accumulate a large amount of L-valine in the culture medium.[83] The L-valine production was maximum at the sub-optimal level of L-leucine for growth. At the higher levels, L-leucine inhibited the L-valine production. Because the selected strain Rα-88 was proved to be a leucine auxotroph, the effect of L-leucine concentration on the production of L-histidine and L-valine by this mutant was investigated with a medium containing molasses (15% as glucose concentration) as carbon source (Fig. 1). The L-histidine production was maximum when the L-leucine concentration was suboptimal for growth (20 μg/ml), and was only slightly reduced by the addition of it at 50 μg/ml, the level which gave the maximal growth and strongly inhibited the L-valine production. The addition of L-leucine at 300-500 μg/ml distinctly inhibited the L-histidine production together with the L-valine production.

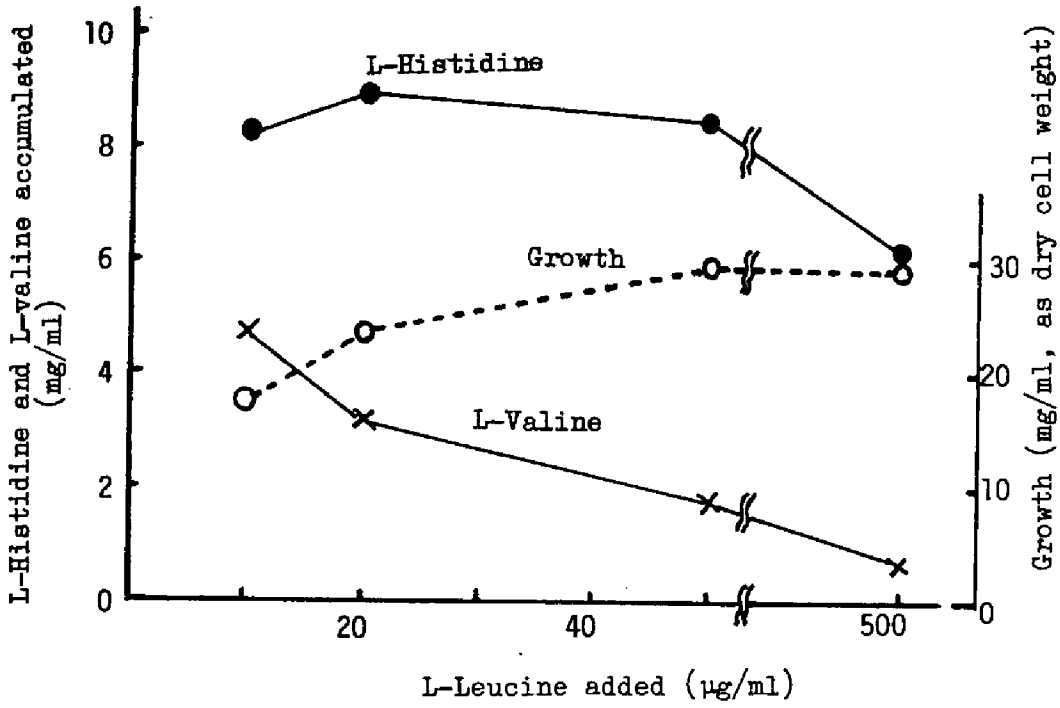


Fig. 1 Effect of L-Leucine on L-Histidine Production by a Leucine Auxotroph Derived from C. glutamicum KY-10260

Fermentation was carried out in a 500-ml Sakaguchi flask containing 40 ml of fermentation medium basically comprising of the ingredients shown in the legend of Table IV except that yeast extract was omitted.

DISCUSSION

Based on the concept of metabolic interlock between L-histidine and aromatic amino acids in some other microorganisms, the author expected a high production of L-histidine by C. glutamicum mutants carrying aromatic amino acid-auxotrophy in addition to histidine analog-resistance. However, TA-resistant mutants derived from a tyrosine auxotroph, a phenylalanine auxotroph and a trytophan auxotroph of a C. glutamicum strain, did not produce such a high level of L-histidine as expected. The level was lower than or the same with that of C. glutamicum KY-10260, a non-auxotrophic TRA-resistant mutant.

L-Histidine production test with 164 auxotrophic derivatives of KY-10260 revealed the distinct superiority of a leucine auxotroph Rα -88 over the parent strain with respect to L-histidine productivity. Excessive amount of L-leucine inhibited the L-histidine production by Rα -88. These results are of interest in the light of observations that L-leucine recovered the growth-inhibition by TA in Brevibacterium flavum[31] and that L-leucine production by an L-leucine-producing mutant of C. glutamicum is remarkably enhanced by the addition of histidine-auxotrophy to the mutant.[84] It seems to be possible that there may exist some inter-pathway-relationship between L-histidine and L-leucine in "coryneform glutamic acid-producing bacteria".

SUMMARY

Corynebacterium glutamicum mutants carrying both auxotrophy and histidine analog-resistance were derived by a mutagenic treatment, and their L-histidine productivity was compared with that of a triazolealanine (TRA)-resistant L-histidine producer, C. glutamicum KY-10260. As a result, a leucine auxotrophic TRA-resistant mutant, Rα -88 was selected out of 164 auxotrophic derivatives of KY-10260. It produced L-histidine at a distinctly higher concentration than the parent strain under every condition tested. The concentration reached 11 mg/ml or 5.8% (w/w) of the initial sugar. Addition of an excessive amount of L-leucine to the medium inhibited the L-histidine production together with the by-production of L-valine by this mutant. Thiazolealanine-resistant mutants derived from a tyrosine auxotroph, a phenylalanine auxotroph and a tryptophan auxotroph gave the same or lower production in comparison with KY-10260.

Section 3. L-Histidine Production by *Corynebacterium glutamicum* Mutants, Multi-resistant to Analogs of Histidine, Tryptophan, Purine and Pyrimidine

INTRODUCTION

At present, the involvement of 5-phosphoribosylpyrophosphate (PRPP) in the biosynthesis of L-histidine has been known.[29,56,60,85-87] The first step of L-histidine biosynthesis is a condensation of adenosine 5'-triphosphate (ATP) and PRPP. The biosynthetic pathway leading to ATP involves the purine pathway. PRPP also serves as a skeleton upon which the heterocyclic ring of purine is built.

The biosynthesis of PRPP in *Salmonella typhimurium*[88,89] and *Escherichia coli*[89,90] is inhibited by adenosine 5'-diphosphate (ADP) and less effectively by other purine nucleotides, pyrimidine nucleotides and L-tryptophan at the step of PRPP synthetase (EC 2.7.6.1). The formation of PRPP synthetase of *S. typhimurium* has recently been revealed to be repressed by uridine nucleotides.[91,92] The adenine nucleotide synthesis in bacterial systems is regulated by purine derivatives. PRPP amidotransferase (EC 2.4.2.14), the first enzyme of the purine pathway, from *Aerobacter aerogenes*[93] is inhibited by guanosine 5'-monophosphate (GMP), adenosine 5'-monophosphate (AMP) and guanosine 5'-triphosphate (GTP), and less effectively by ADP and inosine 5'-monophosphate

(IMP). In Bacillus subtilis,[94] the enzyme inhibition by guanosine nucleotides is much less than that of the adenine nucleotides, and IMP has been shown to be ineffective. The inhibitory effect of 5-amino-1-(5'-phosphoribosyl)-4-imidazolecarboxamide (AICAR) and of ATP on this enzyme is also described in S. typhimurium[86] and Brevibacterium ammoniagenes,[114] respectively. Adenylosuccinate synthetase (EC 6.3.4.4), the first enzyme in the AMP-specific pathway, is inhibited by guanosine 5'-diphosphate (GDP) and GMP, and less effectively by AMP and ADP in E. coli.[95] The GDP inhibition is much less than that of AMP and ADP in B. subtilis.[96] Formation of the enzymes of the common pathway for the purine nucleotide synthesis is repressed by the derivatives of both adenine and guanine or by either of them, in S. typhimurium,[97] A. aerogenes,[97,98] E. coli,[86] B. subtilis[99,100] and B. ammoniagenes.[114] The formation of the adenylosuccinate synthetase is repressed by adenine derivatives in B. subtilis[100, 101] and in A. aerogenes.[98]

As already described (Chapter IV, Section 1), histidine analog-resistant mutants of Corynebacterium glutamicum accumulated 6-7 mg/ml [4-4.7 % (w/w) of the initial sugar] of L-histidine in the culture medium, and the high production of L-histidine by these mutants was explained in terms of the lack of end product regulation on the histidine pathway. If, in these mutants, the histidine pathway is so extensively relieved of the end product regulation that the biosynthesis of such precursors of L-histidine as PRPP and adenine nucleotides limits the L-histidine production, the increase of the

biosynthesis of the precursors will lead an increased production of L-histidine. Moreover, it has generally been recognized in bacterial systems that the mutations causing resistance to a structural analog of a normal metabolite involve a mutation causing overproduction of the metabolite or its related metabolites.

Based on these facts, the author attempted the improvement of L-histidine productivity of an L-histidine-producing mutant, C. glutamicum KY-10260, by letting it be resistant successively to each analog of purine, pyrimidine, histidine and tryptophan.

MATERIALS AND METHODS

Microorganisms. C. glutamicum KY-10260, an L-histidine producer which was derived in the previous section (Section 1 of this chapter) as TRA-resistant from a wild-type strain ATCC 13761 was used as the original parent strain of all the analog-resistant mutants described in this study.

Mutagenesis. Every mutagenesis was performed by the treatment of bacterial cells with N-methyl-N'-nitro-N-nitrosoguanidine (NTG). Prior to the mutagenesis, the sensitivity of a parent strain to an analog was tested by streaking the cells on a minimal agar medium (Table I in Section 1 of this chapter) supplemented with the analog at various concentrations from 50 to 8000 μg/ml and incubating at 30°C. The analog-resistant mutant was selected on the agar medium containing the analog at twice to ten times the minimal

inhibitory concentration of the parent strain.

Culture method. Fermentation was carried out in a 250-ml Erlenmeyer flask for 92~96 hr by the manner described in the previous section (Section 1 of this chapter). Compositions of the fermentation medium were described in the legend of each table and figure.

Analysis. L-Histidine and the bacterial growth were measured by the method described in the previous section (Chapter II, Section 1). Adenine was measured spectrophotometrically from the absorbancy at 260 nm after paperchromatographic separation in isobutyric acid— 28% NH_4OH (100:20 by volume) as solvent and elution with hot water.

Reagents. Sources of the reagents used are as follows: 6-mercaptoguanine and 8-azaguanine from Nutritional Biochemicals Co.; 6-mercaptopurine, dithiouracil, 6-methylpurine and 5-methyltryptophan from Sigma Chemical Co.; 4-thiouracil and 3-amino-1,2,4-triazole from Tokyo Kasei Kogyo Co., Ltd.; DL-2-thiazolealanine and DL-1,2,4-triazole-3-alanine from Eli Lilly & Co.; tubercidin from Calbiochem. 2-Fluoroadenine was synthesized by Mr. Teranishi of Kyowa Hakko Kogyo Co., Ltd. according to the method of Montgomery.[102)]

RESULTS

1. Improvement of L-histidine productivity by the addition of single purine analog-resistance.

The biosynthetic pathway leading to adenine nucleotides[86, 93,101,103] and PRPP[88-90] in bacterial systems is known to be regulated by purine nucleotides. Furthermore, such purine analogs as 6-mercaptopurine (MP), 6-mercaptoguanine (MG) and 8-azaguanine (AG) are shown to mimic some of the above-described regulatory action of the normal metabolites after conversion to the nucleotides corresponding to each analog.[104,105] Moreover, these analogs have been shown to inhibit the growth of a C. glutamicum strain.[106] 2-Fluoroadenine (FA) has been shown to be a feedback inhibitor of purine biosynthesis in E. coli.[107]

Based on these informations, the author expected that some mutation causing C. glutamicum resistance to these analogs would allow the overproduction of precursors of L-histidine, PRPP and ATP, and the overproduction of them would result in an increased production of L-histidine in an L-histidine producer. At first, attempts were made to derive the mutants resistant to these analogs from a TRA-resistant L-histidine producer, C. glutamicum KY-10260. Resistants were selected on the agar media containing one of these analogs at the concentration indicated in Table I. The concentrations used were much higher than 62.5 μg/ml, the level which completely inhibited the growth of the parent strain KY-10260 in a preliminary experiment. Two hundred and fifty mutants thus

obtained were tested for L-histidine productivity in a medium containing 15% (as glucose concentration) cane molasses as carbon source. Table I shows the L-histidine production by four strains, each gave the highest yield of L-histidine in each class of the mutants selected on the agar medium containing different analog. As can be seen, an MG-resistant mutant MG-15, an AG-resistant mutant AG-121 and an FA-resistant mutant FA-43 produced 7.4, 7.0 and 6.7 mg/ml of L-histidine, respectively. These values were higher than that (5.2 mg/ml) given by the parent strain KY-10260. Out of them, strain

Table I. L-Histidine Production by Purine Analog-resistant Derivatives of *C. glutamicum* KY-10260

Class of mutants	No. of mutants tested	Best L-histidine producer in each class		
Resistant to		Strain No.	L-Histidine produced (mg/ml)	Growth (O.D.660 x 1/100)
2-Fluoroadenine(1000μg/ml)	73	FA-43	6.7	0.53
6-Mercaptoguanine(1000μg/ml)	80	MG-15	7.4	0.64
6-Mercaptopurine(200μg/ml)	50	MP-22	5.5	0.64
8-Azaguanine(500μg/ml)	47	AG-121	7.0	0.76
(Parent)		KY-10260	5.2	0.62

Fermentation medium: 15% (as glucose concentration) cane molasses, 4.5% $(NH_4)_2SO_4$, 0.15% KH_2PO_4, 0.05% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.3% urea, 3% $CaCO_3$; pH 7.2.

MG-15 was selected as parent strain for the further mutation to improve the L-histidine productivity in the following experiment. The L-histidine productivity of the MP-resistant mutants was comparable with that of KY-10260, but was lower than that of MG-15, AG-121 and FA-43.

2. By-production of adenine by FA-resistant derivatives of KY-10260

In the experiment described above, it was found that all the FA-resistant derivatives of KY-10260 accumulated 0.5~2.0 mg/ml of adenine in addition to L-histidine in the culture medium. Identification as adenine was obtained from the agreement of its Rf-values in paperchromatography (Table II) and its UV-absorption spectrum with those of authentic adenine. In comparing the amount of adenine with that of L-histidine produced by each FA-resistant mutant, it was noted, as shown in Fig. 1, that low production of L-histidine tended to accompany high production of adenine and that high production of L-histidine did not always accompany low production of adenine. For example, FA-21, FA-60 and FA-69 which were three lowest L-histidine producers, gave the highest yield of adenine. Strain FA-43 which gave the highest yield of L-histidine accumulated 1.2 mg/ml of adenine, the amount of adenine being comparable with that (1.2 ~ 1.3 mg/ml) given by strain FA-18 or FA-45 which exhibited low L-histidine productivity. Adenine was also produced by FA-resistant derivatives of a wild-type strain C. glutamicum ATCC 13761, from which all the mutants described here were originated, as reported elsewhere.[108)]

Table II. Paperchromatographic Identification of Adenine Produced by 2-Fluoroadenine-resistant Mutants (Rf-values)

Solvent system*	Substance produced by the mutant	Authentic adenine
A	0.60	0.60
B	0.12	0.12
C	0.94	0.95

*) Solvent system (ratio by volume): A, n-propanol-0.2N NH_4OH (3:1); B, isopropanol-CH_3COONa-$(NH_4)_2SO_4$ saturated solution (2:19:79); C, isobutyric acid-1 N NH_4OH (5:1).

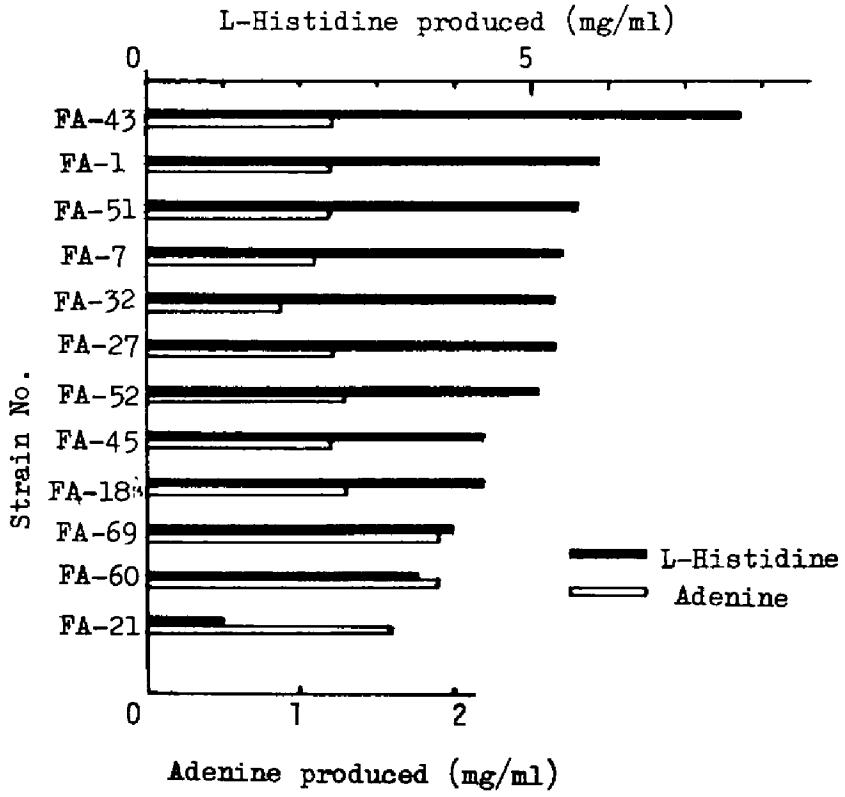


Fig. 1 Relation between the L-Histidine Titers and Adenine By-production by 2-Fluoroadenine-resistant Derivatives of C. glutamicum KY-10260

3. Improvement of L-histidine productivity by the addition of double purine analog-resistance

An MG-resistant mutant, MG-15 gave the highest yield of L-histidine in the previous experiment (Table I). The improvement of the L-histidine productivity of this mutant was attempted by a further addition of AG-resistance marker to the mutant, because the emergence of AG-resistance in KY-10260 was rather effective on the L-histidine production (Table I). As a result of an L-histidine production-test with 32 mutants of such MG- and AG-resistants, a mutant MGAG-16 gave the highest yield (7.8 mg/ml) of L-histidine. This mutant was used as the parent strain for the further mutation to improve the L-histidine productivity in the next experiment.

4. Improvement of L-histidine productivity by the addition of pyrimidine analog-resistance

The biosynthesis of PRPP is inhibited by cytosine nucleotides and uridine nucleotides at the step of PRPP synthetase in S. typhimurium[88,89] and E. coli.[90,125] The formation of PRPP synthetase in S. typhimurium is repressed by uridine nucleotides.[91,92] The uridine nucleotides can be derived from uracil via "salvage pathway", and the cytosine nucleotides derivedfrom the uridine nucleotides.[85,109] Moreover, some pyrimidine-analogs cause the inhibition of bacterial growth by mimicing the regulatory effect of normal pyrimidine nucleotides.[104,105,109] As a fact, pyrimidine analogs, 4-thiouracil (4TU), 6-azauracil (6AU) and dithiouracil (DTU, 2,4-dithiopyrimidine) inhibited the growth of an L-histidine producer MGAG-16 which gave the highest yield of L-histidine

Table III. Distribution of Pyrimidine Analog-resistant Derivatives of MGAG-16 with respect to L-Histidine Productivity

Class of mutants	No. of mutants tested	No. of strains producing L-histidine in amount (mg/ml) of					
		6>	6-7	7-8	8-9	9-10	10-11
Resistant[a] to							
6-Azauracil	69	6	7	17	26	13	0
4-Thiouracil	28	1	4	5	11	4	3
Dithiouracil	40	2	6	10	14	7	1

Fermentation medium: The same as that in the footnote of Table I except that 0.75% meat extract was added.

a) Mutants were derived in the presence of the analog at 500 µg/ml.

Table IV. L-Histidine Production by Best L-Histidine Producer in Each Class of Pyrimidine Analog-resistant Derivatives of C. glutamicum MGAG-16

Class of mutants	Strain No.	L-Histidine produced (mg/ml)	Growth (O.D.660 X1/100)
Resistant to			
6-Azauracil	6AU6-37	9.9	0.62
4-Thiouracil	4TU6-2	10.3	0.53
Dithiouracil	DTU6-2	10.1	0.63
(Parent)	MGAG-16	6.3	0.59

Details of the experiment were the same as those in the footnote of Table III.

in the preceding experiment, as well as wild-type strain C. glutamicum ATCC 13761.

Based on these facts and the similar assumption as that described for the derivation of purine analog-resistant mutants, the author expected a high production of L-histidine by the mutants resistant to these pyrimidine-analogs. Such mutants were derived from the MGAG-16. An L-histidine production test with 137 mutants in all of 6AU-resistant, 4TU-resistant and DTU-resistant mutants revealed that more than half of these pyrimidine analog-resistant mutants produced L-histidine at the higher levels than 8 mg/ml, as shown in Table III. As can be seen in Table IV, a 6AU-resistant mutant 6AU6-37, a 4TU-resistant mutant 4TU6-2 and a DTU-resistant mutant DTU6-2 produced 9.9, 10.3 and 10.1 mg/ml of L-histidine, respectively. Among them, strain 4TU6-2 was employed as the parent strain for a further mutagenesis in the next experiment.

5. Improvement of L-histidine productivity by the addition of 6-methylpurine-resistance and tubercidin-resistance

In the preceding experiment, a 4TU-resistant mutant 4TU6-2 exhibited a high productivity of L-histidine. For a further improvement of its L-histidine productivity, the author sought some purine-analogs which were inhibitory to the mutant. A purine analog, 6-methylpurine (MEP) and a nucleoside antibiotic, tubercidin (Tu) were found to completely inhibit the mutant's growth at 250 and 4000 μg/ml, respectively. Therefore, the mutants resistant to each of these analogs were derived from 4TU6-2. As shown in Table V,

the amount of L-histidine produced by these mutants was 10 mg/ml at best. This value was comparable with that given by the parent strain 4TU6-2 in the preceding experiment (Table IV), though it was higher than that given by the parent strain in this experiment. Based on this result, an MEP-resistant mutant TUM-39 listed in Table V was used as parent strain in a further mutagenesis in the next experiment.

Table V. L-Histidine Production by 6-Methylpurine-resistant Derivatives and Tubercidin-resistant Derivatives of C. glutamicum 4TU6-2

Class of mutants	No. of mutants tested	Best L-histidine producer in each class		
		Strain No.	L-Histidine produced (mg/ml)	Growth (O.D.660 X1/100)
Resistant to				
6-Methylpurine(2000μg/ml)	60	TUM-39	10.0	0.52
Tubercidin (8000μg/ml)	50	TUT-44	8.9	0.51
(Parent)		4TU6-2	7.8	0.62

Fermentation medium used was the same as that in the footnote of Table III.

6. Improvement of L-histidine productivity by increasing the resistance to TRA

FA was a unique purine-analog in that the emergence of resistance to it in an L-histidine producer KY-10260 caused the by-production of adenine (Fig. 1). In this case, a high production of L-histidine still accompanied the production of

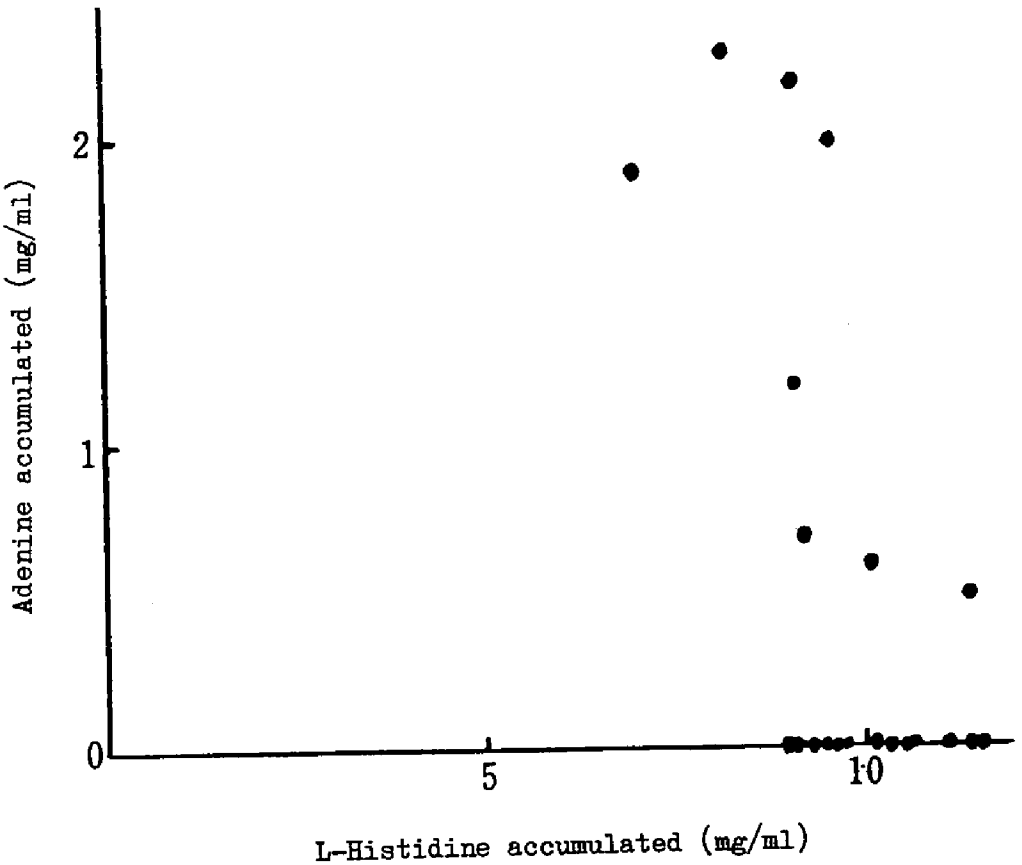


Fig. 2 Relation between L-Histidine Titers and Adenine By-production by 2-Fluoroadenine-resistant Derivatives of C. glutamicum TUM-39

a considerable amount of adenine. Consequently, if the adenine nucleotide is used for the biosynthesis of L-histidine without being excreted into the medium as adenine, the L-histidine production will increase. This was expected in the

case of an FA-resistant mutant simultaneously carrying 4TU-resistance, if the latter resistance allowed the overproduction of PRPP and the excess of PRPP was used for the L-histidine biosynthesis coupling with ATP. These situations prompted us to the derivation of FA-resistant mutant from TUM-39 which had been derived from a 4TU-resistant mutant 4TU6-2 in the preceding experiment. An FA-resistant derivative TUMF-20 thus obtained produced 11.4 mg/ml of L-histidine. This value was rather higher than that (10.7 mg/ml) given by the parent strain TUM-39. Eight out of thirty FA-resistant derivatives produced adenine as well as L-histidine. As shown in Fig. 2, high L-histidine producers by-produced smaller amount of adenine than low L-histidine producers.

The growth of TUM-39 was delayed in the presence of such histidine-analogs[29] as 2-thiazolealanine (TA), TRA or 3-amino-1,2,4-triazole (AMT). Therefore, it seemed still possible in TUM-39 that L-histidine production is limited by the residual end product regulation on the histidine pathway. When 10^7 NTG-treated cells of TUM-39 per plate were spread on a minimal agar medium supplemented with 200 μg/ml of TRA, 88 colonies appeared with a slight background growth on the entire agar-surface. When a similar experiment was performed with the medium supplemented with both of TA and AMT each at the concentration of 1000 μg/ml, 10 colonies appeared with similar background growth. In the presence of either TA or AMT, only the slight growth over the entire agar-surface was observed, but no distinct colonies appeared. The author isolated the colonies, which rapidly grew in the presence of these histidine-analogs, as the mutants highly resistant to the analogs. Two

Table VI. L-Histidine Production by Histidine Analog-resistant Derivatives of *C. glutamicum* TUM-39

Class of mutants	No. of mutants tested	Strain No.	L-Histidine produced (mg/ml)	Growth (O.D.660 X1/100)
Increased resistance to				
Triazolealanine (200 μg/ml)	76	TUMT-24	12.2	0.63
Thiazolealanine (1000 μg/ml) plus aminotriazole (1000 μg/ml)	10	TUMA-1	10.1	0.39
(Parent)		TUM-39	10.7	0.57

The details of the experiment were the same as those in the footnote of Table III.

strains which gave the highest yield of L-histidine among the mutants, and the data on their L-histidine productivity are listed in Table VI. A mutant TUMT-24 selected from the mutants which grew rapidly in the presence of TRA produced a distinctly higher amount (12.2 mg/ml) of L-histidine than its parent strain(10.7 mg/ml).

7. Effect of cane molasses-sucrose ratio on L-histidine production

As already described, cane molasses stimulated the L-histidine production by a TRA-resistant L-histidine producer, KY-10260 (Section 1 of this chapter), and its leucine auxotrophic derivative (Section 2 of this chapter). At the ap-

Table VII. Effect of Molasses-Sucrose Ratio on L-Histidine Production by C. glutamicum TUMT-24

Percentage of molasses*	L-Histidine produced (mg/ml)	Growth (O.D.660X1/100)
0	6.4	0.38
37.5	13.4	0.41
50.0	11.2	0.55
59.0	10.8	0.50
100.0	10.8	0.49

*) The percent sugar concentration of molasses to a total sugar used is given.

The molasses-sucrose ratio was adjusted by combining the following two media in different ratios. Medium I: Sucrose 15%, KH_2PO_4 0.2%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, urea 0.2%, $(NH_4)_2SO_4$ 4%, $FeSO_4 \cdot 7H_2O$ 0.001%, $MnSO_4 \cdot 4H_2O$ 0.001%, meat extract 0.75%, thiamine·HCl 500 µg/liter, biotin 200 µg/liter and $CaCO_3$ 3%. Medium II: Cane molasses 15% (as glucose concentration), $MgSO_4 \cdot 7H_2O$ 0.05%, $(NH_4)_2SO_4$ 4%, K_2HPO_4 0.1%, KH_2PO_4 0.2%, urea 0.2%, meat extract 0.75% and $CaCO_3$ 3%.

propriate molasses-sucrose ratio, the level of the L-histidine production by these mutants became maximum. Therefore, the effect of molasses concentration on L-histidine production by TUMT-24 which gave the highest yield of L-histidine in the preceding experiment was investigated. It was done with media containing 15% sugar comprising of cane molasses and sucrose in different ratios. As can be seen in Table VII , the mutant produced 13.4 mg/ml [8.9% (w/w) of the initial sugar] of L-histidine under the condition where the molasses percent is 37.5%.

Table VIII. Distribution of 5-Methyltryptophan-resistant Derivatives of C. glutamicum TUMT-24 with Respect to L-Histidine Productivity

Exp. No.	No. of mutants tested	No. of strains producing L-histidine in amount (mg/ml) of						
		<9	9-10	10-11	11-12	12-13	13-14	14-15
I (molasses medium[a])	88	35	18	21	9	5	0	0
II(molasses-sucrose medium[b])	79	18	4	9	15	15	10	8

a) The molasses medium was the same as that in the footnote of Table III.

b) The molasses-sucrose medium was composed of 6% (as glucose concentration) cane molasses, 9% sucrose, 0.2% KH_2PO_4, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.2% urea, 4% $(NH_4)_2SO_4$, 0.0006% $FeSO_4 \cdot 7H_2O$, 0.0006% $MnSO_4 \cdot 4H_2O$ and 0.75% meat extract, biotin 120 µg/l, thiamine·HCl 1500 µg/l.

5-Methyltryptophan-resistant mutants were derived in the presence of 500 or 1000 µg/ml of the analog.

8. Improvement of L-histidine productivity by the addition of tryptophan analog-resistance

The tryptophan inhibition of PRPP synthetase is known with crude extracts from S. typhimurium[88] and E. coli[125]. Based on a similar assumption as that described for the derivation of purine analog-resistant mutants, the author expected the high production of L-histidine by the mutant carrying resistance to 5-methyltryptophan (5MT) which has

Table IX. L-Histidine Production by Representative 5-Methyltryptophan-resistant Derivatives of C. glutamicum TUMT-24

Exp. No.	Strain No.	L-Histidine produced (mg/ml)	Growth (O.D.660 X1/100)
I (molasses medium)	AT-83	12.6	0.44
	AT-32	12.2	0.64
	TUMT-24 (Parent)	9.6	0.61
II(molasses-sucrose medium)	AT-83	15.0	0.44
	AT-85	14.6	0.40
	TUMT 24 (Parent)	12.6	0.42

The details of the experiment were the same as those in the footnote of Table VIII.

been shown to be a regulator of the aromatic pathway of bacteria.[110~112])

Such mutants were derived from TUMT-24 which gave the highest yield of L-histidine in the preceding experiment, and cultured at first in a fermentation medium containing 15% (as glucose concentration) cane molasses. As can be seen in Tables VIII and IX (Exp. I), 5 out of 88 such mutants produced 12 ~ 12.6 mg/ml of L-histidine. These levels of L-histidine were comparable with or higher than those given by the parent strain TUMT-24 in the preceding experiment (12.2 mg/ml) and in this experiment (9.6 mg/ml, Exp. I in Table IX), respectively.

Seventy-nine of the 5MT-resistant mutants were cultured again in a medium containing 6% (as glucose concentration) cane molasses and 9% sucrose as carbon sources. The concentration [40% (w/w) of the total sugar] of molasses used was near the optimal one (37.5%) for the L-histidine production by the parent strain TUMT-24. Eight of them produced 14 ~ 15 mg/ml of L-histidine (Exp. II in Table VIII); the values were distinctly higher than those given by the parent strain in the preceding experiment (13.4 mg/ml, Table VII) and in this experiment (12.6 mg/ml, Exp. II in Table IX). The highest yield was given by AT-83 also under this condition, and the level of L-histidine produced by this mutant reached 15 mg/ml or 10% (w/w) of the initial sugar.

DISCUSSION

The L-histidine productivity of a TRA-resistant L-histidine producer, C. glutamicum KY-10260 could be improved in a stepwise manner by the successive additions of such resistance markers as purine analog-resistance, pyrimidine analog-resistance, high resistance to histidine-analog and tryptophan analog-resistance to the mutant. The genealogy of AT-83, a finally selected mutant strain, and of the mutants which were employed as parent strain at each step of improvement is shown in Fig. 3, together with their L-histidine productivity. The improvement of the L-histidine productivity in each step was rather minor, but as a total a finally selected mutant strain, AT-83 produced approximately twice as much L-histidine

as the original L-histidine producer KY-10260. Amongst the steps, the addition of 4TU-resistance caused most significant increase in the L-histidine productivity. The distribution pattern of 4TU-resistant mutants with respect to L-histidine productivity (Table III) also supports the significance of 4TU-resistance marker.

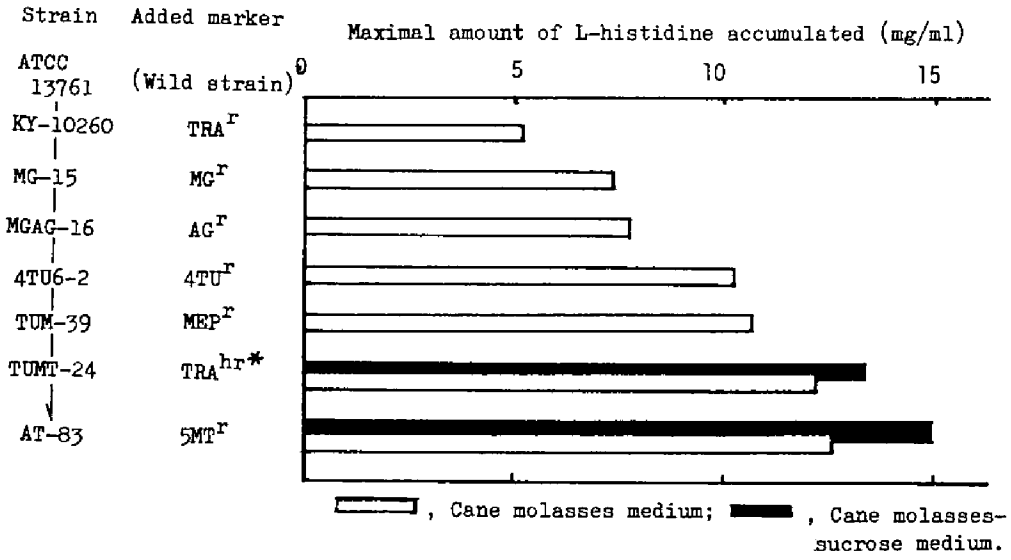


Fig. 3. Increase in L-Histidine Productivity by the Successive Additions of Purine, Pyrimidine, Histidine and Tryptophan Analog-resistance to C. glutamicum KY-10260

*) High resistance to TRA.

The rational of the improvements were based on the speculation that the regulatory mechanism on L-histidine biosynthesis and related biosynthesis known in some other microorganisms[29,53,56,60,85,87,113] would be applicable to C. glutamicum. These are supplying of increased amount of PRPP and adenine nucleotide for L-histidine biosynthesis by releasing the feedback regulation on their biosynthesis in the regulatory mutant obtainable as an end product-analog resistant. The improvement by increasing the resistance to TRA could be explained in terms of a further release of end product regulation in histidine pathway in consequence of the additional mutation at the regulatory site[29] other than the original one in KY-10260.

FA-resistant mutants derived from both histidine-producing mutants and a wild-type strain (ATCC 13761[108]) of C. glutamicum accumulated a large amount of adenine in the culture medium. This is explained in terms of the lack of feedback regulation on the biosynthesis of adenine nucleotides, overproduction and decomposition of the nucleotides and excretion in the form of adenine base.

SUMMARY

The L-histidine productivity of an L-histidine producer Corynebacterium glutamicum KY-10260, which was derived as a 1,2,4-triazole-3-alanine (TRA)-resistant mutant, was improved by successive additions of such markers as purine analog-resistance, pyrimidine analog-resistance, histidine analog-resistance and tryptophan analog-resistance to the mutant. A selected mutant AT-83, multi-resistant to 6-mercaptoguanine, 8-azaguanine, 4-thiouracil, 6-methylpurine, TRA and 5-methyltryptophan, accumulated twice as much L-histidine as KY-10260 in the culture medium. The level of L-histidine production by this mutant reached 15 mg/ml or 10 % (w/w) of the initial sugar in the medium containing 6 % (as glucose concentration) cane molasses and 9 % sucrose as carbon sources. 2-Fluoroadenine-resistant mutants produced adenine in addition to L-histidine.

Section 4. Feedback-resistant Phosphoribosyl-ATP Pyrophosphorylase in L-Histidine Producing Mutants of *Corynebacterium glutamicum* and Its Significance for L-Histidine Production

INTRODUCTION

Moyed and Magasanik[56] first described the involvement of adenosine 5'-triphosphate (ATP) and ribosylphosphate in L-histidine biosynthesis. It was established with enteric bacteria that imidazoleglycerol phosphate (IGP), a known intermediate[51] for L-histidine biosynthesis, is produced from ribose 5-phosphate, the amide nitrogen of L-glutamine, and N-1, C-2 portions of the adenine ring of ATP.

Genetic and enzyme studies on *Salmonella typhimurium* by Ames, Hartman, Martin and their coworkers[29] have provided many informations on the biosynthetic pathway for L-histidine and control mechanisms of the pathway. The first step of the pathway is a condensation of 5-phosphoribosyl-1-pyrophosphate (PRPP) and ATP to form N-1-(5'-phosphoribosyl) adenosine triphosphate (phosphoribosyl-ATP) and pyrophosphate. The reaction is mediated by N-1-(5'-phosphoribosyl) adenosine triphosphate : pyrophosphate phosphoribosyltransferase (phosphoribosyl-ATP pyrophosphorylase, EC 4.2.1c), and is subject to feedback inhibition by L-histidine.[60] Certain mutants of *S. typhimurium* resistant to a histidine-analog, 2-thiazolealanine (TA), were found to have a pyrophosphorylase resistant to feedback inhibition. This property offered an

explanation for the L-histidine-excretion by TA-resistant mutants of this microorganism[66] and Escherichia coli.[67,115] The histidine pathway is also under feedback repression control.[29,60,116] Mutants resistant to 1,2,4-triazole-3-alanine (TRA) of S. typhimurium have been found to be relieved of the repression control.[29,70]

As described in the previous section (Chapter IV, Section 1), Corynebacterium glutamicum mutants which were derived as resistants to either TA or TRA accumulated a large amount of L-histidine in the culture medium. A mutant strain obtained from one of the above TRA-resistant mutants by successive additions of purine analog-resistance, pyrimidine analog-resistance and increased resistance to TRA, produced twice as much L-histidine as the original L-histidine producer (Chapter IV, Section 3).

In the present investigation, some properties of the pyrophosphorylase of some L-histidine producers of C. glutamicum and related strains were investigated to clarify the mechanism of L-histidine production by the L-histidine producers.

MATERIALS AND METHODS

Microorganisms. C. glutamicum ATCC 13761, a wild-type strain, and its derivatives were used in this study. KY-10234 (ATCC 21339) is defective in histidinol dehydrogenase (EC 1.1.1.23) and produces L-histidinol in the culture medium (Chapter II, Sections 1 and 2). KY-10260 and KY-10261 are L-histidine producers derived from ATCC 13761 as a TRA-resistant and a

TA-resistant, respectively. KY-10260 has been clarified by a growth experiment to be resistant also to TA. KY-10522 (TUMT-24) is a derivative of KY-10260, being improved in the L-histidine productivity through successive additions of 6-mercaptoguanine-resistance, 8-azaguanine-resistance, 4-thiouracil-resistance, 6-methylpurine-resistance and increased resistance to TRA. This mutant strain produced twice as much L-histidine as KY-10260. S. typhimurium hisG46hisO1242 was a gift of Dr. B.N.Ames. This mutant strain is defective in phosphoribosyl-ATP pyrophosphorylase and produces constitutively other histidine enzymes.[46]

Preparation of enzyme extract. Dialyzed cell-free extracts used in this study as enzyme preparation were prepared by the method described in the previous chapter (Chapter II, Section 2) with a slight modification. The cells of C. glutamicum were cultured in an Erlenmeyer flask containing 20 ml of the growth medium. In the case of C. glutamicum KY-10234, a histidine auxotroph, the cells were grown on 0.026~0.52 mM L-histidine. The cells of S. typhimurium hisG46hisO1242 harvested were frozen at -20°C without washing and used for each enzyme preparation.

Phosphoribosyl-ATP pyrophosphorylase. The enzyme activity was assayed principally according to the "method b" of Martin et al.[49] Phosphoribosyl-ATP was converted to N-(5'-phospho-D-1'-ribulosyl-formimino)-5-amino-1-(5''-phosphoribosyl)-4-imidazolecarboxamide (BBM III), the forth intermediate in L-histidine biosynthesis,[56,117] and the BBM III was spectrophotometrically determined. An excess of S. typhimurium

hisG46hisO1242 extract[46] provided an excess of the enzyme required to convert phosphoribosyl-ATP to BBM III. The complete assay mixture contained 1.5 ml of tris (hydroxymethyl) aminomethane-HCl buffer (pH 8.5) supplemented with 0.02M $MgCl_2$ and 0.3M KCl, 0.15 ml of ATP solution, 0.15 ml of PRPP solution, 0.05 ml of the extract of S. typhimurium hisG46hisO1242, enzymes and water to 3 ml. Solutions of L-histidine, histidine-analogs, NH_4Cl and L-glutamine were added to the mixture in place of water according to the purpose of experiment. The control contained no ATP. The reaction was started by the addition of PRPP solution, and the absorbancy at 290 nm of the mixture was spectrophotometrically followed for 5 min in a cuvette with 10-mm light path, using Hitachi Colorimeter Model 139. The reaction temperature was maintained using Komatsu Solidate Type SPR-S apparatus.

Determination of imidazole and arylamine. Periodate oxidation method described by Ames et al. for IGP[60] was applied for the determination of the imidazole formed, with the modification of eight-fold scale of the original method, using imidazole-glycerol as the standard. The butanol layer-extract from the samples gave two peaks, at the wave lengths of 275-280 and 310-315 nm, in the UV-absorption spectrum, like that with authentic imidazoleglycerol. The arylamine formed in enzyme reaction or by acid-hydrolysis of bound diazotizable amine was determined by the modified Bratton-Marshall method of the five-fold scale of the original procedure[60] using 5-amino-4-imidazolecarboxamide riboside as the standard. The absorption spectrum of the chromatophore produced with the samples coincided with that produced with the standard riboside.

Protein determination. Protein was determined by the Folin method[50] as described in the previous section (Chapter II, Section 2).

Reagents. Adenosine 5'-triphosphate·Na_2, L-histidine·HCl, D-erythro-imidazoleglycerol·HCl were the products of Kyowa Hakko Kogyo Co., Ltd. Phosphoribosylpyrophosphate·Na_2·$4H_2O$, DL-1,2,4-triazole-3-alanine·H_2O, DL-α-methylhistidine·2HCl, 5-amino-4-imidazolecarboxamide riboside were purchased from Sigma Chemical Co. DL-2-thiazolealanine was purchased from Cyclo Chemical Co. Bovine albumin (fraction V) was purchased from Armour Pharmacology.

RESULTS

1. Validity of the enzyme assay

The enzyme assay method of Martin et al.[49] employed here for phosphoribosyl-ATP pyrophosphorylase is based on the following informations derived from the studies on S. typhimurium[60] and E. coli.[56] The product of the forward reaction, phosphoribosyl-ATP, is converted to BBM III in the presence of the extract of S. typhimurium hisG46hisO1242. The extract of the S. typhimurium mutant provides in excess the enzymes required to convert phosphoribosyl-ATP to BBM III but is deficient in the pyrophosphorylase. Further metabolism of BBM III is prevented by the deficience of L-glutamine(or NH_4Cl) which is required for its metabolism. BBM III formed is spectrophotometrically followed at 290 nm where this compound

has a molecular extinction coefficient of 9×10^3.[49]

To confirm the validity of the assay, the characterization of the reaction product in our assay system as BBM III was attempted. When a complete assay mixture was incubated for 105 min, the absorbancy at 290 nm increased by 0.562. The value corresponded to the formation of 0.19 μmoles of BBM III per 3 ml calculated on the basis of its molecular extinction

Table I. Estimation of BBM III

I[a]	Amount of BBM III estimated from the increase of absorbancy.	0.19 μmoles
II[b]	Amount of BBM III estimated from the formation of AICAR.	0.21 μmoles

An enzyme reaction mixture containing 5×10^{-3}M ATP, 3.3×10^{-4}M PRPP, extract of C. glutamicum ATCC 13761 (2.3 mg as protein) and other ingredients of "complete assay mixture" described in MATERIALS AND METHODS section was incubated for 105 min at 37°C.

a) A molar extinction coefficient for BBM III of 9×10^3 at pH 8.5 and 290 nm was used in converting absorbancy to μmoles.
b) Assayed as 5-amino-4-imidazolecarboxamide riboside, after acid-hydroltsis by the method of Ames et al. Without hydrolysis, the value was 0.04 μmoles.

coefficient. The BBM III was converted to 5-amino-1-(5'-phosphoribosyl)-4-imidazolecarboxamide (AICAR) by mild acid-hydrolysis[60] and the AICAR formed was determined by Bratton-Marshall reaction (Table I). The amount of BBM III exactly corresponds to the amount (0.21 μmoles/3 ml) of diazotizable

amine determined as AICAR. The formation of BBM III was confirmed further by another experiment. The experiment is based on that the extract of S. typhimurium converts BBM III to equimolar amounts of AICAR and IGP in the presence of L-glutamine or NH_4Cl.[60,117] As shown in Table II, the amounts of these metabolites were approximately equimolar. The estimation of IGP and AICAR was accomplished by the methods described by Ames et al.[60] for each compound. Omitting ATP from the reaction mixture used in the conversion of BBM III to AICAR and IGP did not result in such metabolites. Thus the metabolites were confirmed to be the product of

Table II. Effect of Ammonium Chloride and L-Glutamine on the Formation of Imidazoleglycerol and AICAR

ATP added (M)	Ammonium source added (M)	Products[a] (μmoles/3ml) Imidazole	AICAR
0	0	0.00	0.00
$5X10^{-3}$	0	0.04	0.06
$5X10^{-3}$	NH_4Cl ($3.33X10^{-2}$)	0.32	0.33
$5X10^{-3}$	L-Glutamine($6.7X10^{-3}$)	0.25	0.37

The reaction mixtures, each containing $3.3X10^{-4}$M PRPP, extract of C. glutamicum ATCC 13761 (2.3 mg as protein), indicated levels of ATP and other ingredients of the complete assay mixture were incubated at 37°C for 75 min, and incubation was continued for 30 min after the addition of the indicated level of NH_4Cl or glutamine.

a) Determined by the methods described in "MATERIALS AND METHODS".

enzyme reaction. A small amount of these metabolites accumulated even in the absence of L-glutamine or NH_4Cl; this may be ascribed to the contamination of some nitrogen sources from excessive enzyme extract used in this experiment.

These results together with the results of the succeeding experiment on the requirements for enzyme reaction, support that the change of absorbancy of the assay mixture employed here corresponds to the amount of BBM III formed and reflects the pyrophosphorylase activity in C. glutamicum strains.

2. Requirements for pyrophosphorylase reaction

Fig. 1 shows that the wild-type enzyme of KY-10234 requires ATP, PRPP and Mg^{++} for its pyrophosphorylase reaction, like that of S. typhimurium.[60] Omission of the extract of S. typhimurium hisG46hisO1242 from the complete assay mixture resulted in 60% reduction of the activity. Increasing the amount of the S. typhimurium extract did not increase the activity. These results support that the activity determined in the assay system employed here expresses the pyrophosphorylase activity of C. glutamicum. A slight but significant increase in absorbancy was observed without addition of ATP, PRPP or C. glutamicum enzyme. Based on this result, the enzyme activity was estimated by subtracting the absorbancy in the absence of ATP from the total absorbancy.

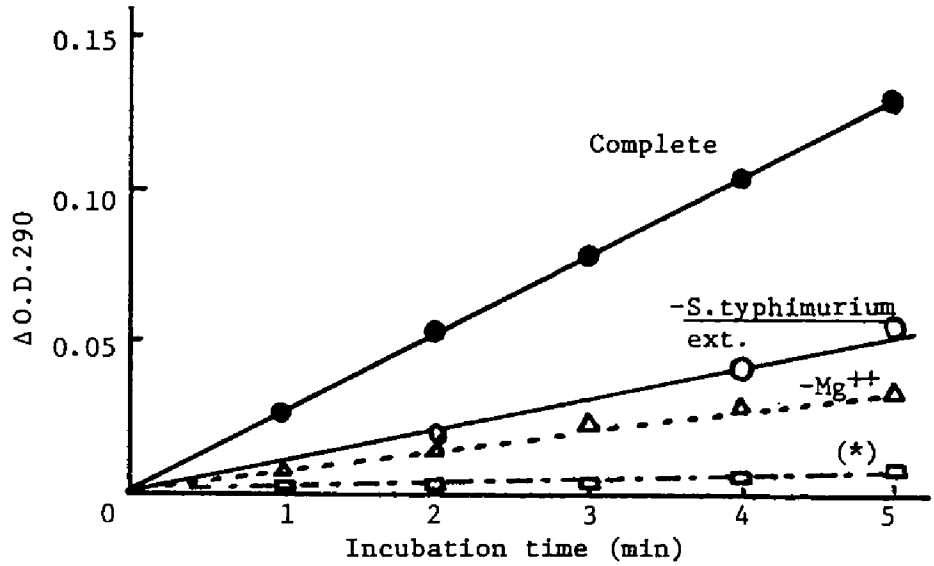


Fig. 1 Requirements for PR-ATP Pyrophosphorylase Reaction
ATP; $5X10^{-3}$M; PRPP: 10^{-3}M; temperature: 37°C.
(*): -ATP, -PRPP or -C. glutamicum enzyme.

3. Dependence on enzyme concentration

Dependence of the pyrophosphorylase activity on enzyme concentration was confirmed with wild-type enzyme at 30°C or 37°C, with the PRPP concentration of 3.3×10^{-4}M or 10^{-3}M. As shown in Fig. 2, the response was linear upto the absorbancy of 0.19 at 37°C with 10^{-3}M PRPP. With 3.3×10^{-4} PRPP, the response was linear upto the absorbancy of 0.1 at both 30°C and 37°C.

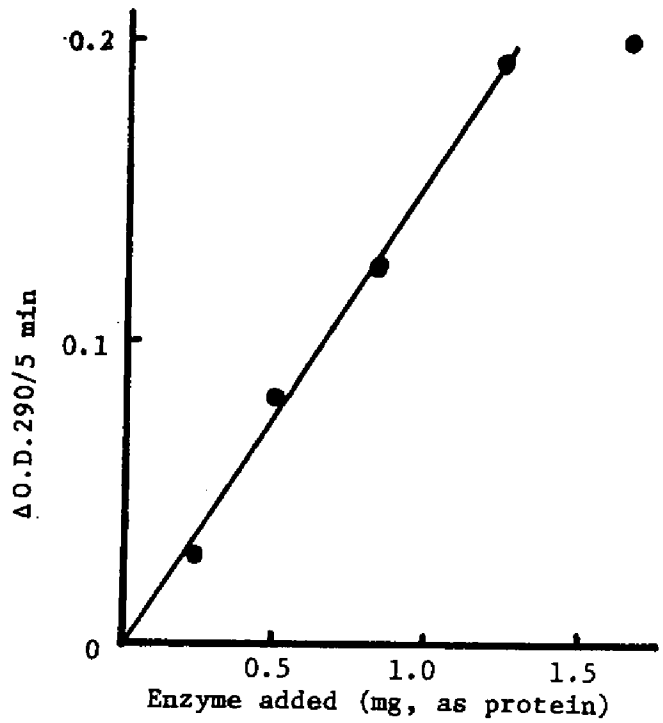


Fig. 2 Dependence on Enzyme Concentration
ATP: 5×10^{-3}M; PRPP: 10^{-3}M; temperature: 37°C;
enzyme: C. glutamicum KY-10234.

4. Effect of temperature

Effect of temperature on the wild-type pyrophosphorylase activity was investigated at a PRPP concentration of 3.3×10^{-4}M, a standard concentration of PRPP used for the study on the pyrophosphorylase of S. typhimurium.[49] The activity increased with the increase of temperature up to 42.5°C where the highest activity was attained, as shown in Fig. 3.

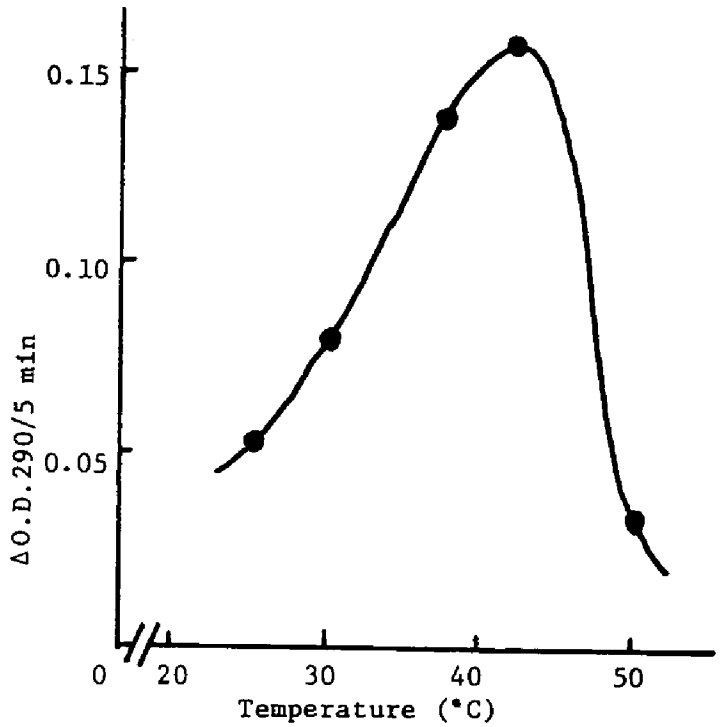


Fig. 3 Effect of Temperature
ATP: $5X10^{-3}$M; PRPP: $3.3X10^{-4}$M; enzyme: C. glutamicum KY-10234.

5. Inhibition by L-histidine

The pattern of L-histidine-inhibition of the pyrophosphorylase of wild-type enzyme from KY-10234 was compared with that of the enzyme from L-histidine-producing histidine analog-resistant mutants, KY-10260, KY-10261 and KY-10522. The enzyme activity assay was carried out with 3.3×10^{-4}M PRPP

and at 30°C, optimal temperature for L-histidine production by the above L-histidine-producers. As shown in Fig. 4, the activity of the wild-type enzyme reduced by 50% or 96% in the presence of 3.3×10^{-4}M or 10^{-3}M L-histidine, respectively. In contrast, the activity of the enzyme from every L-histidine-producer was not inhibited by the same concentrations of L-histidine and inhibited as effectively as the wild-type enzyme at 100-fold higher L-histidine concentrations. L-Histidine-inhibition on the wild-type enzyme was investigated also with 10^{-3}M PRPP, the concentration near that producing the substrate saturation as shown in Fig. 7. In this case, the inhibitory effect of L-histidine was rather less than the one with 3.3×10^{-4}M PRPP, i.e., 6×10^{-4}M L-histidine produced 50% inhibition. Almost the same L-histidine inhibition-pattern was obtained with 30°C and 37°C (Fig. 5).

In the inhibition studies described above and in the succeeding section, the following facts should be noted. As described in the experiment shown in Fig. 1, omission of the S. typhimurium hisG46his01242 extract from the complete assay mixture resulted in the reduction of the activity. This means that the extract of the S. typhimurium mutant stimulates the conversion of phosphoribosyl-ATP, which is formed by the action of the pyrophosphorylase in the extract of C. glutamicum, to BBM III. The remainder of the activity (40%, in the case of the experiment shown in Fig. 1), is believed to be due to the over-all reaction of BBM III-forming system (including pyrophosphorylase) of C. glutamicum. There has been no evidence in C. glutamicum that the BBM III-forming enzymes other than the pyrophosphorylase are not inhibited by L-histidine or its

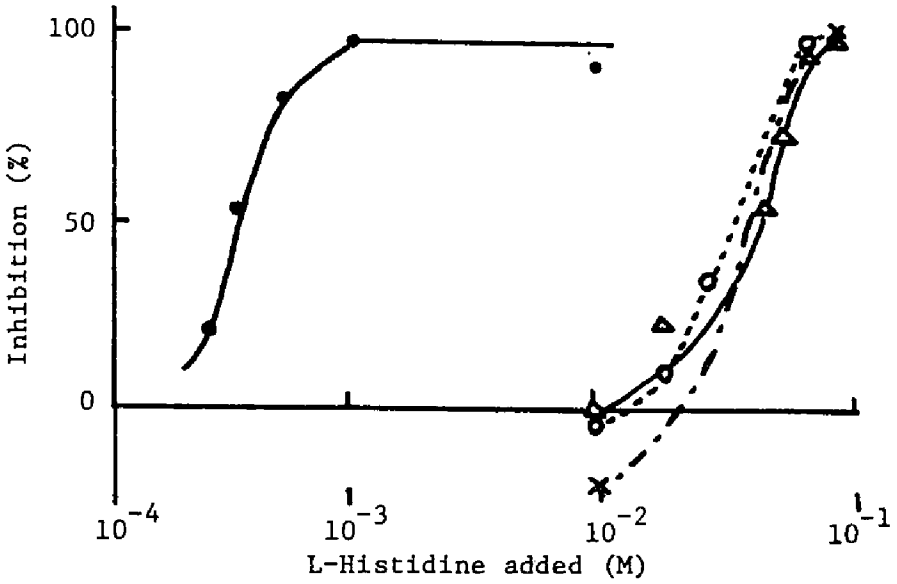


Fig. 4 Effect of L-Histidine on the Activity of PR-ATP Pyrophosphorylase from Various C. glutamicum Strains

Enzyme: ●——●, KY-10234; o···o, KY-10260; Δ——Δ, KY-10261; ×-·-×, KY-10522
ATP: 5×10^{-3} M; PRPP: 3.3×10^{-4} M; temperature: 30°C.

analogs. In this respect, it is questionable that the inhibition pattern determined in the coupled assay system employed here is an exact reflection of the nature of the pyrophosphorylase of C. glutamicum, though this question might vanish in future in reference to the insensitivity of the BBM III-forming enzymes other than the pyrophosphorylase to the feedback inhibition in S. typhimurium. Because of this insensitive nature of the conversion step of phosphoribosyl-ATP to BBM III in S. typhimurium, on the other hand, at least the effect on

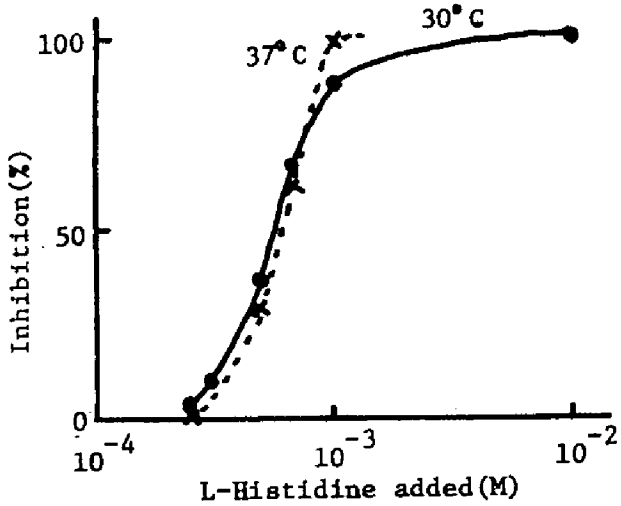


Fig. 5 Effect of Temperature on L-Histidine-inhibition
ATP: $5X10^{-3}$M; PRPP: 10^{-3}M; enzyme:KY-10234.

the fraction of the activity of the enzyme, which was reduced by eliminating the Salmonella extract from the reaction mixture as shown in Fig. 1 (about 60% of the complete system), can directly be ascribed to the effect on the pyrophosphorylase of C. glutamicum in the inhibition studies. For example, under the condition employed in the experiment of Fig. 1, the inhibition over 40% of the total activity doubtelssly involves the inhibition against the pyrophosphorylase of C. glutamicum. Under these situations, it is generally concluded that the pyrophosphorylase of C. glutamicum is almost completely inhibited by 10^{-3}M L-histidine but hardly is inhibited by 10^{-4}M L-histidine, and that all the above L-histidine-producers have the pyrophosphorylase 100-fold resistant to the feedback inhibition.

6. Inhibition by histidine-analogs

The effect of TA and α-methylhistidine(MH), known inhibitors of the pyrophosphorylase of S. typhimurium,[119] on the pyrophosphorylase of C. glutamicum was investigated. As shown in Table III, the activity of wild-type enzyme from KY-10234 reduced by 50% or 90% in the presence of 3 X 10^{-3}M or 10^{-2}M each histidine-analog, respectively. MH inhibited also the enzymes from histidine analog-resistant mutants, KY-10260, KY-10261 and KY-10522 as effectively as the wild-type enzyme. In contrast, TA did not inhibit and rather stimulated the reaction of the enzymes from these mutants at least at 10^{-2}M.

Table III.

Effect of Histidine-analogs on the Activity of PR-ATP Pyrophosphorylase from Various C. glutamicum Strains

Source of enzyme (Strain No.)	Percent inhibition in the presence* of						
	DL-α-Methylhistidine				DL-2-Thiazolealanine		
	10^{-3}	$2X10^{-3}$	$3X10^{-3}$	10^{-2}	10^{-3}	$3X10^{-3}$	10^{-2}
KY-10234	34	38	46	92	5	46	90
KY-10260		36		99			0
KY-10261		62		100			-28
KY-10522				98			-13

* Molar concentration of L-isomer.
ATP: $5X10^{-3}$M. PRPP: $3.3X10^{-4}$M. Temperature: 30°C.

7. Repression by L-histidine

Attempts were made to clarify whether the pyrophosphorylase formation in C. glutamicum is under the repression control of L-histidine or not.

As exemplified in Table IV, the growth rate of KY-10234, a histidine auxotroph, distinctly reduced when L-histidine concentration in the growth medium was lowered from 0.52 mM to 0.026 or 0.052 mM. The growth rate with 0.52 mM L-histidine was fairly close to that of wild-type strain ATCC 13761. The pyrophosphorylase levels were determined for the cells of KY-10234 grown with the above three concentrations of L-histidine. As can be seen in Table V, the cells grown with the limited amounts of L-histidine were derepressed for the enzyme ; the specific activity of the pyrophosphorylase was 2.8- or 1.6-fold higher in the cells grown with 0.026 or 0.052 mM L-histidine, respectively, compared with the cells grown on 0.52 mM L-histidine. In another experiment, three-fold de-repression was noted with the cells grown with 0.052 mM L-histidine.

Table IV. Growth Response of a Histidine Auxotroph, KY-10234 to L-Histidine

Strain No.	L-Histidine added(mM)	Growth (O.D.660 X/40)
KY-10234	0.520	0.22
	0.052	0.07
	0.026	0.04
	0	0.00
ATCC 13761 (Wild-type)	0	0.28

Incubation time: 19 hr.

Table V Repression by L-Histidine of PR-ATP Pyrophosphorylase Formation in a Histidine Auxotroph, KY-10234

L-Histidine added to the medium (mM)	Δ O.D.290/5 min /mg protein	Relative activity
0.520	0.044	1.0
0.052	0.069	1.6
0.026	0.124	2.8

ATP: 5×10^{-3}M. PRPP: 3.3×10^{-4}M. Temperature: 37°C.

8. Levels of the pyrophosphorylase in C. glutamicum strains

A large number of TRA-resistant mutants have been isolated from S. typhimurium.[29] These included regulatory mutants generally giving more than three-fold derepression of histidine operon.[120,121] Because KY-10260 and KY-10522 had been selected in the presence of TRA, they might have derepressed levels of the pyrophosphorylase. Accordingly, the specific activity of the pyrophosphorylase of these mutants was determined in comparison with those of wild-type strain ATCC 13761 and a TA-resistant L-histidine-producer, KY-10261. As shown in Table VI, the specific activity of the pyrophosphorylase from KY-10260 was not so distinctly higher than that from ATCC 13761 and KY-10261 as expected. In contrast, the pyrophosphorylase activity was two-fold elevated in KY-10522 compared with the wild-type strain.

Table VI. Levels of PR-ATP Pyrophosphorylase in Various *C. glutamicum* Strains

Strain No.	ΔO.D.290/5 min /mg protein	Relative activity
ATCC 13761	0.047	1.0
KY-10260	0.062	1.3
KY-10261	0.053	1.1
KY-10522	0.094	2.0

ATP: $5X10^{-3}$M. PRPP: 10^{-3}M. Temperature: 37°C.

9. Substrate kinetics

The apparent Km for PRPP (with 5 x 10^{-3}M ATP) or for ATP (with 2 x 10^{-3}M PRPP) was obtained from a Lineweaver-Burk plot, and was 6.67 x 10^{-4}M (Fig.7) or 4.9 x 10^{-4}M(Fig.6), respectively.

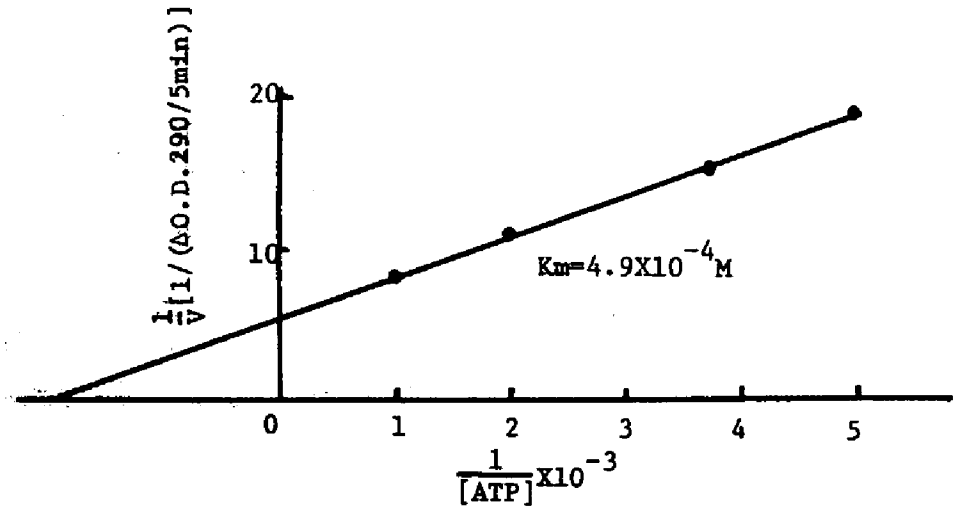


Fig. 6 Determination of Km for ATP
PRPP:$2x10^{-3}$M; temperature:37°C; enzyme:KY-10234

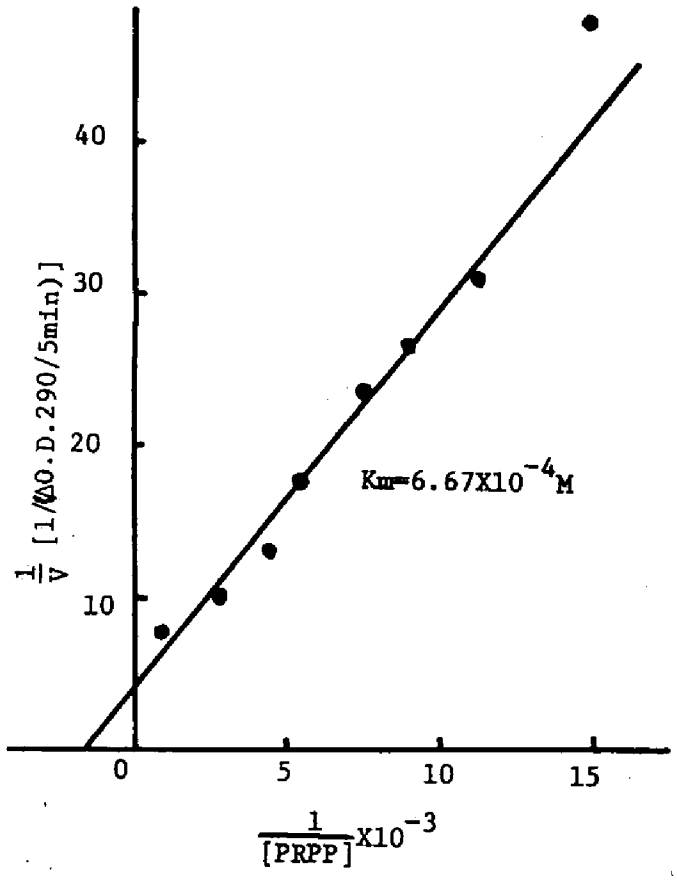


Fig. 7 Determination of Km for PRPP

ATP: 5×10^{-3}M; temperature: 37°C;
enzyme: KY-10234.

DISCUSSION

As described above, phosphoribosyl-ATP pyrophosphorylase of the wild-type C. glutamicum was strongly inhibited by L-histidine. By the addition of 3 ~ 6 x 10^{-4}M or 10^{-3}M L-histidine, the reaction rate reduced by 50% or 95 ~ 100%, respectively ; 10^{-4}M L-histidine hardly inhibited the reaction. Strictly speaking, the inhibition-pattern thus determined may be a reflection of the L-histidine-inhibition to both the pyrophosphorylase and the BBM III-forming system of C. glutamicum, as illustrated in the section of RESULTS. However, this inhibition to the BBM III-forming system probably represents the inhibition to the pyrophosphorylase. This presumptive conclusion is based on the fact that, in L-histidine biosynthetic pathway of other microorganisms, only pyrophosphorylase is known to be subject to L-histidine-inhibition and the other enzymes concerned in BBM III-synthesis are not. Thus the inhibition by L-histidine observed in the experiments of Figs. 4 and 5 may directly reflects the sensitivity of the pyrophosphorylase of C. glutamicum to L-histidine. Even at present, it can be concluded that the pyrophosphorylase of C. glutamicum is inhibited almost completely by 10^{-3}M L-histidine but hardly inhibited by 10^{-4}M L-histidine. This shows that the pyrophosphorylase of this microorganism is less sensitive to L-histidine-inhibition than the enzyme of S. typhimurium[66,118,119,122] which is inhibited 50% by 5 ~ 8 x 10^{-5}M L-histidine under the similar conditions as described here.

TA and MH are well-known histidine-analogs.[29] Each of them inhibited the wild-type pyrophosphorylase of C. glutamicum

ten-fold less effectively than L-histidine. A similar correlation has been recognized between the effects of L-histidine and these histidine-analogs on the S. typhimurium pyrophosphorylase.[119,122] In E. coli,[67,123] however, the BBM III-forming system is hardly inhibited by MH, though TA inhibits the system. In these respects, the C. glutamicum pyrophosphorylase resembles that of S. typhimurium.

Limited supplying of L-histidine to a histidine auxotroph, KY-10234, resulted in three-fold derepression of the formation of the pyrophosphorylase at maximum. The derepression level is much lower in comparison with that in S. typhimurium[124] and E. coli[46], in which more than ten-fold derepression was noted. However, the result obtained here indicates the histidine pathway of C. glutamicum is under feedback repression control at least at the first step.

Two L-histidine producers, KY-10260 and KY-10261, each derived as a TRA-resistant and a TA-resistant from a wild-type strain ATCC 13761, had the feedback inhibition-resistant pyrophosphorylase. The mutant enzyme was 100-fold resistant to the inhibition of L-histidine and TA. However, the enzyme formation was not appreciably derepressed in both mutants. Such repressed formation of pyrophosphorylase in KY-10260 is different from the case of S. typhimurium, where mutations to TRA-resistance involved the regulatory mutation causing release of the repression control.[29,70,120,121] In any way, one reason for L-histidine production by KY-10260 and KY-10261 may reside in the loss of feedback inhibition control on the pyrophosphorylase.

Another L-histidine producer, KY-10522 (TUMT-24) had two-fold derepressed level of the pyrophosphorylase. This mutant had been derived from KY-10260 by the addition of the markers, purine analog-resistance, pyrimidine analog-resistance, and increased resistance to TRA, and has the increased L-histidine-productivity. Though it is not verified whether the additional TRA-resistant marker or other resistant markers described above caused the derepression, the lack of the repression control offers an explanation for the increased L-histidine-productivity of KY-10522.

Effect of MH on the pyrophosphorylase reaction was unique in that this histidine-analog inhibited the feedback-resistant pyrophosphorylase as effectively as the wild-type enzyme. The mechanism of this phenomenon is not known, but it seems to be probable that the inhibition site of MH on the pyrophosphorylase may be different from that of L-histidine and TA.

SUMMARY

Phosphoribosyl-ATP pyrophosphorylase of two L-histidine-producers of Corynebacterium glutamicum, each selected as a 2-thiazolealanine (TA)-resistant and a 1,2,4-triazole-3-alanine (TRA)-resistant, was found to be 100-fold resistant to L-histidine-inhibition in comparison with wild-type enzyme. It was also resistant to the inhibition by TA, but still as sensitive as the wild-type enzyme to the inhibition by α-methyl-histidine. Formation of the pyrophosphorylase in these mutants

was not significantly derepressed. However, two-fold derepression was noted with a further improved L-histidine producer KY-10522, a derivative of the above TRA-resistant. KY-10522 is an improved strain in L-histidine-productivity through the additions of resistance markers including increased resistance to TRA. Phosphoribosyl-ATP pyrophosphorylase of KY-10522 was found to be resistant to the feedback inhibition, like its parent strain.

Chapter V. Conclusion

In the present study, the author investigated two fermentation processes for L-histidine production.

The first one is a two-steps process, namely production of the imidazole intermediates of the histidine pathway with bacterial histidine auxotrophs and the microbial conversion of the imidazoles into L-histidine with other microorganisms.

A histidine auxotrophic mutant of Corynebacterium glutamicum was found to produce L-histidinol, the terminal intermediate of the histidine pathway, at the concentration of 11 mg (dihydrochloride) per ml in the culture medium. Enzymic study with dialyzed cell-free extract revealed that the L-histidinol producer is defective in histidinol dehydrogenase, the enzyme converting L-histidinol into L-histidine. Moreover, it was proved that phosphoribosyl-ATP pyrophosphorylase, the first enzyme of the histidine pathway, of this microorganism is regulated by both of feedback inhibition and repression by L-histidine. Based on these facts, the L-histidinol production was explained in terms of the block of the metabolism of L-histidinol due to the defect in histidinol dehydrogenase, the release of the feedback regulation under the L-histidine-limitation, and the excretion of the L-histidinol into the culture medium.

When the later-phase culture of the L-histidinol producer was mix-cultured with an Escherichia coli strain, 4 mg/ml of L-histidine was accumulated with the reduction of L-histidinol. The microbial conversion of L-histidinol into L-histidine is believed to be attained by the action of histidinol dehydrogenase

of the E. coli within or outside of the cells. The cell-permeability of the L-histidinol and L-histidine and the scarcity of L-histidine-degradation may be relevant to the peculiar nature of the E. coli strain.

A histidine auxotroph of Brevibacterium ammoniagenes produced 13 mg (as monohydrochloride) per ml of imidazoleglycerol in the culture medium. However, the microbial conversion of this imidazole into L-histidine was unsuccessful.

The second process investigated for L-histidine production depended on the use of the regulatory mutants which were derived as histidine analog-resistant mutants. It was found that 2-thiazolealanine(TA)-resistant mutants and/or 1,2,4-triazole-3-alanine(TRA)-resistant mutants derived from C. glutamicum, Arthrobacter citreus, Brevibacterium fluvum, Bacillus megaterium, Bacillus subtilis and Nocardia globerula accumulated a considerable amount of L-histidine in the culture medium. Of these L-histidine producers, C. glutamicum KY-10260 which was derived as a TRA-resistant was investigated for the culture condition. It was most important for the L-histidine production to use cane molasses as carbon source and a comparably high amount of ammonium salts as nitrogen source. Thus the amount of L-histidine accumulated reached a level of 6~7 mg/ml of 4~4.7 (w/w) of the initial sugar with a medium containing 15 % (as glucose concentration) cane molasses and 4.5 % $(NH_4)_2SO_4$.

Various kinds of auxotrophic mutants were derived from the L-histidine producer KY-10260, and their L-histidine productivity was compared with that of the parent strain. As the result, a leucine auxotrophic derivative Rα-88 was selected

out of 164 auxotrophic derivatives of KY-10260. The mutant produced L-histidine at a distinctly higher concentration than the original L-histidine producer under every condition tested. The L-histidine production by Ra-88 reached 11 mg or 5.8 % (w/w) of the initial sugar. The L-histidine production was inhibited by L-leucine, which suggests that L-histidine biosynthesis of C. glutamicum is regulated by L-leucine through a certain unclarified mechanism.

The L-histidine productivity of C. glutamicum KY-10260 could also be improved in a stepwise manner by the successive additions of such resistant markers as purine analog-resistance, pyrimidine analog-resistance, high resistance to histidine-analog and tryptophan analog-resistance to the mutant. The improvement of the productivity in each step was rather minor, but a finally selected mutant AT-83 produced approximately twice as much L-histidine as the original L-histidine producer KY-10260. The level of L-histidine production by AT-83 reached 15 mg/ml or 10 % (w/w) of the initial sugar.

To clarify the mechanism of the L-histidine production by the above C. glutamicum mutants, some properties of phosphoribosyl-ATP pyrophosphorylase were investigated with the dialyzed extracts from the L-histidine producers and the related C. glutamicum strains. The wild-type enzyme was almost completely inhibited by 10^{-3}M L-histidine, 10^{-2}M TA and 10^{-2}M α-methyl-histidine. The enzyme formation of a histidine auxotroph was three-fold derepressed at maximum when the mutant was grown under L-histidine-limitation. On the other hand, the mutant enzymes extracted from two L-histidine producers (KY-10261 and KY-10260), each derived as a TA-resistant and a TRA-resistant.

were 100-fold resistant to the inhibition by L-histidine and TA but were still as sensitive as the wild-type enzyme to the inhibition of α-methylhistidine. Formation of the pyrophosphorylase was not derepressed in these L-histidine producers. In contrast, two-fold derepression was noted with a further improved L-histidine producer KY-10522, a mutant multi-resistant to 6-mercaptoguanine, 8-azaguanine, 4-thiouracil, 6-methylpurine and TRA.

These results indicate that the L-histidine productivity of the original L-histidine producer C. glutamicum KY-10260 is due to the feedback inhibition-resistance at least of phosphoribosyl-ATP pyrophosphorylase, and that a partial derepression of the formation of the first enzyme also contributes to the increased L-histidine productivity of the improved L-histidine producers.

The fact that a single-step mutation simultaneously induced both of the high L-histidine productivity and the feedback inhibition-resistance of the first enzyme suggests that the feedback inhibition control on the histidine pathway is mainly exerted on the first enzyme in C. glutamicum. It remains unclear whether the histidine enzymes other than the pyrophosphorylase are under the repression control or not. It is also unclear whether the alteration of the repression control on these enzymes contributes to the L-histidine productivity of the C. glutamicum mutants or not.

β-Imidazolelactic acid, a known metabolite of L-histidine, was by-produced in the L-histidine fermentation. The amount of this imidazole produced was usually less than one tenth of that of L-histidine. A minute amount of this imidazole also

accumulated in the L-histidine-supplemented culture of the parent wild-type strain C. glutamicum ATCC 13761. The scarcity of the L-histidine-degradation may be another reason of the L-histidine production by C. glutamicum mutants. The cell-permeability may also be relevant to the L-histidine production.

In the present study, the author found the accumulation of adenine, imidazoleacetol and glycine, besides the substances described above, in considerable amounts.

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