Kor. J. Appl. Microbiol. Bioeng.
Vol. 14, No. 5, 427 -432(1986)

Genetic regulation for the biosynthesis of glutamate family in *Corynebacterium glutamicum*

Kim In-Ju, Kyung Hee Min and Sae Bae Lee*

Department of Biology, Sookmyung Women's University & *Miwon Co. LTD.

(Received September 22, 1986)

*Corynebacterium glutamicum*에서의 glutamate계 아미노산 생합성의 유전적 조절

金仁珠 · 閔庚喜 · 이세배*

淑明女子大学校 生物学科, *味元주식회사

(1986년 9 월 22일 수리)

The regulation of three ammonia assimilatory enzymes, GDH (glutamate dehydrogenase), GS (glutamine synthetase) and GOGAT (glutamate synthase), has been examined in *C. glutamicum*. Three kinds of arginine auxotrophs blocked in each step of arginine biosynthetic pathway from glutamate were selected as arg 5, arg 6, arg 8. Histidine and tryptophan auxotrophs were also selected because histidine and tryptophan repressed GS biosynthesis in *E. coli*. These strains were cultured on the media containing nitrogen-excess and limited conditions, to compare the specific activities of α-ketoglutarate dehydrogenase (α-KGDH), GDH, GS, GOGAT from the cell-free extracts. These results showed that enzyme levels of α-KGDH and GDH from 3 kinds of arginine auxotrophs, histidine and tryptophan auxotrophs in nitrogen-excess condition and those of GS and GOGAT in nitrogen limited condition were increased compared with opposite condition. The tryptophan and histidine auxotrophs showed higher level of glutamate and glutamine than parental strains and other mutants. it is assumed that the higher levels of α-KGDH and GDH from mutants in nitrogen-excess condition promoted the accumulation of glutamate and glutamine in fermentation broth. The inhibition of GS activities by ADP suggested that GS is regulated by energy charge in *C. glutamicum*. The results with histidine, tryptophan, glycine, alanine, serine and GMP implied that a system of feedback inhibition were effective. The GDH, GS and GOGAT biosynthesis in culture broth was markedly repressed by the nature and kinds of available nitrogen sources such as tryptophan, proline, glycine, alanine, serine and tyrosine.

Since GS plays a role in nitrogen metabolism in bacteria[7,8], it is key enzyme not only in the biosynthesis of glutamine from glutamate and ammonia but also in the regulation of synthesis of several enzymes involved in the metabolism of nitrogenous compounds. It is well known that the cellular level of GS in *E. coli* is controlled by complex mechanisms[11] and the level of GDH or GOGAT is also regulated by ammonia concentration. Since other works outlined above in *E. coli*, comparative studies have been required on the enzymes of another organisms and its control systems.

We have investigated GS of *C. glutamicum* ATCC 13058 because little information is available on the GS of glutamate producing bacteria though they are valuable in the field of glutamate and glutamine production. In this organism, the glutamine used as a substrate for this reaction is produced from glutamate and ammonia by GS. Thus, GS and GOGAT constitute a cyclic, low ammonia assimilatory pathway that

Key words; pathway blocking by mutation, GS, GDH and GOGAT activities, glutamate and glutamine production

can also provide glutamate when ammonia is in excess.

The present paper concerns in the enzyme activities with nutritional conditions and some enzymatic properties. Specially, in addition to GDH, GS, GOGAT enzyme systems, α-KGDH also is investigated because it is a direct enzyme of other branch of glutamate-glutamine pathway. In order to examine the effect on the blocking of branch pathway arg 5, arg 6, arg 8 auxotrophs which were blocked in arginine biosynthetic pathway from glutamate are compared to parent strain on the α-KGDH, GDH, GS, GOGAT production. Histidine and tryptophan auxotrophs which were repressed on GS biosynthesis are also discussed the effect on enzyme synthesis.

Materials and Method

Strain

The strain used throughout this work was *Corynebacterium glutamicum* ATCC 13058. All bacterial mutants obtained from *C. glutamicum* ATCC 13058 are listed in Table 1.

Media

The minimal salt medium has been described[1] and complete medium was Nutrient agar for the isolation of auxotrophs. MMYE medium[2] as a nitrogen excess condition was used for cultivation and M1 medium was composed of 1% (w/v) glucose, 0.05% KH_2PO_4, 0.05% K_2HPO_4, 0.03% $MgSO_4 \cdot 7H_2O$ and 0.02% yeast extract, pH 7.0. For the nitrogen limiting condition, 1mM NH_4Cl and 1mM amino acid required were added to M1 medium to examine their effect on the formation of α-KGDH, GDH, GS, and GOGAT. In order to investigate feedback inhibition of GS and metabolis repression of these enzymes, M2 medium was used for the experiment of metabolic repression of these enzymes and for cultivation of *C. glutamicum* ATCC 13058. M2 medium was the same as M1 medium with the exception of yeast extract, 0.02%[3]. M3 broth media were used for fermentation of glutamate and glutamine production. M3 medium was consisted of 14% glucose, 3% $(NH_4)_2SO_4$, 0.2% Urea, 0.5% $CaCl_2$, 15ml of GLC (Soybean hydrolysate from Miwon Lab.), 0.14% KH_2PO_4, 0.14% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 3% $CaCO_3$, addition of 20mg of $FeSO_4 \cdot 7H_2O$ and $MnSO_4 \cdot 6H_2O$, 10mg of $ZnSO_4$, $NiCl_2$, and K_2CrO_4, 2mg of Thimine-HCl, 5g of Biotin per liter (pH 7.0).

Isolation of mutants

NTG (N-Methyl-N'-Nitro-N-Nitrosoguanidine) was used for the mutagenesis of auxotrophs from *C. glutamicum* ATCC 13058.

Table 1. *Corynebacterium* strains used in this experiments.

Strains	Phenotype	Source
C. glutamicum ATCC 13058		
C. glutamicum KA8	arg 8	*C. glutamicum* ATCC 13058
C. glutamicum KA6	arg 6	〃
C. glutamicum KA5	arg 5	〃
C. glutamicum KT	trp^-	〃
C. glutamicum KH	his^-	〃

*abbreviation
arg 5, 6, 8, : arginine auxotroph
trp^-: tryptophan auxotroph
his^-: histidine auxotroph

Growth condition of the organism and preparation of crude extract

The organism was cultivated aerobically for 24hr at 30°C in 100ml of broth media in 500ml flask. Washed cells were suspended in 0.02M potassium phosphate buffer(pH 7.0) and disrupted in a sonicator for 30 min. Supernant solution after centrifugation at 12,000rpm for 10 min was dialyzed at 5°C overnight against 0.02M potassium phosphate buffer(pH 7.0).

Assay of enzyme activity

α-KGDH: α-ketoglutarate dehydrogenase (α-KGDH) activity was measured by method of ferricyanide reduction assay[4]. Enzyme unit are μmoles of ferrocyanide formed per minute per mg of protein.

GDH and GOGAT: Glutamate dehydrogenase (GDH) and glutamate synthase (GOGAT) activities were measured by following the rate of reduced NADPH oxidation as described[3]. Enzyme units are μmoles of NADPH oxidized per minute per mg of protein.

GS: Glutamine systhethase (GS) was assayed by transferase reaction[5]. The transferase assay solution consisted of 150 *μmol*, Tris-acetate buffer(pH 6.4), 200 *μmol* glutamine, 40 *μmol* KH_2AsO_4, 1.5 *μmol* ADP, 15 *μmol* $MnCl_2$, and 40 *μmol* hydroxylamine in a final volume of 2*ml*. The assay were carried out at 30°C and initiation by the addition of enzyme extracts. The reaction was stopped by the addition of 1ml of a mixture of 20g trichloroacetic acid and 16g ferric chloride in 500ml of 0.5M HCl. The absorbance at 500nm was read after centrifugation. One unit of enzyme activity was defined as 1 *μmol* of γ-glutamylhydroxamate formed per min[5].

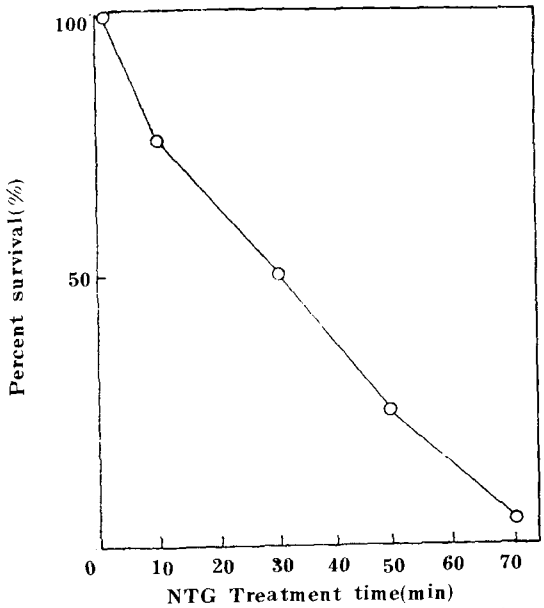

Fig. 1. Survival curve by NTG treatment with final concentration of 200 μg/ml.

Protein determination: Protein content in crude extract was determined by the method of Lowry *et al.*[6] with albumin as a standard.

Treatment of cells with NTG

Incubation of liquid culture was carried out in a reciprocal shaker at 30°C, using 20ml of medium in a 250ml Erlenmyer flask. A expoential culture of strain ATCC 13058 was washed with 0.1M sodium citrate buffer (pH 5.5). The pellet was resuspended in an equal volume of 0.05M trisma-maleic buffer[9]. NTG was added directly to the suspension to give 200μg/ml of final concentration.

After incubation at 30°C for 30 min, cells were washed twice with 0.1M sodium phosphate buffer (pH 7.0), and resuspended in an suspension, it was then serially diluted and plated on Nutrient agar plates.

Result and Discussion

Auxotroph isolation

NTG treatment: In our experiments NTG was added directly to log phase cultures (10^7-10^8 cells/ml) of strain ATCC 13058 growing in broth. Throughout this experiment, the mutagenic activity of NTG was determined by measuring the yield of induced auxotrophic mutants. The auxotrophs were selected by taking cells from logarithmic phase, and treating them for 30 min with NTG in buffer at pH 6.5.(Fig.1). NTG is a potent mutagen because it induces a high frequency

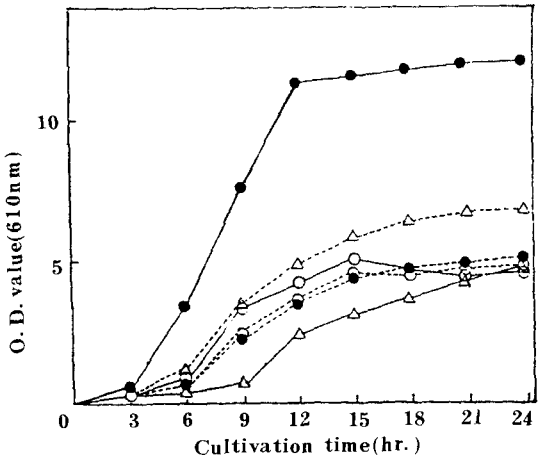

Fig. 2. Growth curve of the *C. glutamicum* ATCC13058(● — ●), KA 48(○ — ○), KA6(● ··· ●), KA5(○ ··· ○), KT(△ ··· △), and KH(△ — △). Cells were grown on MMYE medium.

of mutations at doses which result in little killing. Thus cells are treated under conditions in which there is 50% survival[14].

Auxotroph isolation

Among auxotrophs, isolates were identified as to their growth factor requirements of histidine (19 isolates), tryptophan (10 isolates) and arginine (34 isolates). The other remaining auxotrophs did not respond to only one kind of growth factors, and may represent cells which suffered two

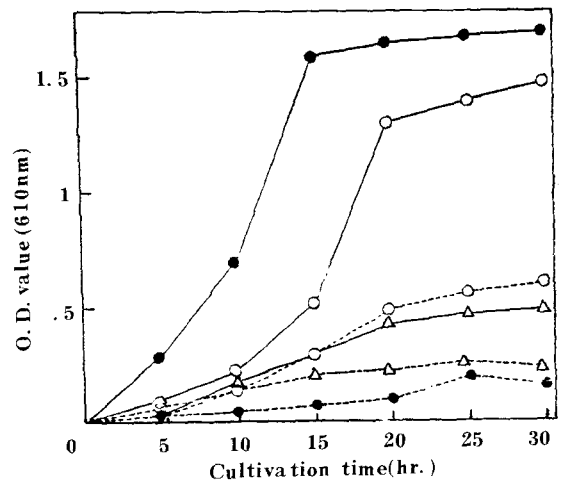

Fig. 3. Growth curve of the *C. glutamicum* ATCC13058(● — ●),KA8(○ — ○), KA6(● ··· ●), KA5 (○ ··· ○), KT(△ ··· △), and KH(△ — △). Cells were grown on M1 medium contained with 1mM NH_4-Cl and supplemented amino acids.

Table 2. Specific activities of α-KGDH, GDH, GS, GOGAT from several mutants grown on M1 medium.

Enzymes / Strains	α-KGDH (×10⁻²)	GDH (×10⁻²)	GS (×10⁻²)	GOGAT (×10⁻²)
C. glutamicum ATCC 13058	4.0	14.0	228	11.7
C. glutamicum KA8	4.8	14.6	102	5.8
C. glutamicum KA6	7.8	17.4	60	9.0
C. glutamicum KA5	12.8	8.1	80	3.8
C. glutamicum KT	2.2	28.9	80	8.4
C. glutamicum KH	3.2	14.2	130	7.2

All enzyme levels are represented as specific activities and the units are μmoles of product formed per minute per milligram of protein.

or more independent auxotrophic mutants. In this experiment by NTG, it is assumed that although mutagenic frequencies were higher than those of UV treatment, the one disadvantage for the use of NTG as a mutagen is that mutants would be mutated at more than one site. This result has also been independently confirmed by Adelberg *et al.*[9]. The arg 5 auxotroph corresponding to acetylornithinase, arg 6 auxotroph to ornithine transcarbamoylase, and arg 8 auxotroph to argininosuccinatelyase defective mutants were identified respectively. There is a branch pathway for arginine biosynthesis from glutamate of main pathway. Thus 3 kinds of argnine auxotrophs were isolated. Histidine and tryptophan showed inhibitory effect to GS activity[10] were also selected.

Growth Curve

In order to compare the growth rate for nitrogen source, cells of arginine, histidine and tryptophan auxotrophs were inoculated into media with nitrogen rich and limiting conditions (Fig. 2 and Fig. 3).

As a nitrogen rich condition, MMYE medium was used for cell cultivation. In this experiment, parental strain ATCC 13058 were more higher growth rate than other amino acid auxotrophs.

Growth rate for nitrogen limiting condition was used M1 medium with 1mM NH_4Cl and the amino acids. Representative growth curve for these mutants are presented in Fig. 3. The growth rate of arginine auxotroph, arg 8, was higher than that of other auxotrophs. Arginine auxotroph, arg 6, and tryptophan auxotroph were lower growth rate under the nitrogen limiting condition. Compare with other works, the growth rate of parental strain usually grows well other than auxotrophs required growth factor.

α-KGDH, GDH, GS, GOGAT activities

Effect of nitrogen limitations on enzyme synthesis: In order to examine enzyme levels for mutant strains which are blocked at the different steps, arg⁻, his⁻ and trp⁻ strains were grown in glucose minimal medium with limited ammonia content. For the nitrogen limited condition, 1 mM of NH_4Cl was contained in culture medium because mutants are required at least low concentration of nitrogen source for their growth.

Table 2 shows the results of enzyme assays for these cultures. Although the GS level of parental strain in ammonium limited condition is elevated in these experiments as that of other mutants. It is clear that the increased GS is coincident with a decrease level of GDH in each strain. An observation reported here is that the GDH activities of several auxotrophs increase during nitrogen excess condition, indicating that this enzyme is regulated by the availability of arginine, tryptophan, and histidine as amino acid source. However, although the GS specific activities of mutant strains are reduced in the nitrogen limited conditions compared with parental strain, it is also same tendency in specific activities of GOGAT. On the other hand, the specific activities of GDH in mutant strains such as arg 5, arg 6, and arg 8 auxotrophs were increased as well as those in α-KGDH.

Effect of nitrogen excess condition in culture medium on GDH, GS, α-KGDH and GOGAT synthesis:

Table 3. Specific activities of α-KGDH, GDH, GS, GOGAT from several mutants grown on MMYE medium.

Enzyme / Strains	α-KGDH (×10⁻²)	GDH (×10⁻²)	GS (×10⁻²)	GOGAT (×10⁻²)
C. glutamicum ATCC 13058	7.9	15.6	162	7.3
C. glutamicum KA8	21.9	16.8	61	11.3
C. glutamicum KA6	15.2	21.3	21	9.1
C. glutamicum KA5	65.6	11.6	12	2.4
C. glutamicum KT	2.1	33.6	11	8.0
C. glutamicum KH	31.3	15.9	36	3.1

All enzyme levels are represented as specific activits and the units are μmoles of product formed per minute per milligram of protein.

Table 4. Specific activities of α-KGDH, GDH, GS and GOGAT from several strains grown on nitrogen excess and limited conditions.

Enzyme specific activities \ Nitrogen condition	Nitrogen excess condition	Nitrogen limited condition
α-KGDH	⇧	↓
GDH	⇧	↓
GS	↓	⇧
GOGAT	↓	⇧

To compare the enzyme level, *C. glutamicum* ATCC 13058 and several auxotrophs were cultured in MMYE medium which contained 0.1% yeast extract and 0.3% urea to be nitrogen excess condition.

As shown in Table 3, specific activities of GDH and α-KGDH in three arginine auxotrophs, histidine and tryptophan auxotrophs were higher than those in parent strain. On the other hand, specific activities of GS in several auxotroph strains were reduced in the nigrogen excess condition even though the GDH levels in same strains are substantially elevated. This tendency in specific activities of GS in nitrogen excess condition was the same as those of GOGAT.

Comparing with Table 2 in nitrogen limited condition and Table 3 nitrogen excess condition, GS and GOGAT levels in nitrogen limited condition were higher than those in nitrogen excess condition. However, GDH and α-KGDH level were appeared in inverse proportion to GS and GOGAT levels in nitrogen limited and excess conditions.

As summarized in Table 4, therefore, this result showed that GDH and α-KGDH levels were higher in nitrogen excess condition whereas GS and GOGAT lower in nitrogen limited condition. Thus at nitrogen excess condition, the result of higher GDH and α-KGDH levels were assumed that α-KGDH will accumulate succinyl CoA in TCA cycle. It was agreed with *E. coli* system that specific activities of GDH in nitrogen excess condition were higher whereas those of GS and GOGAT in nitrogen limited condition lower[13].

Table 5. Glutamate and glutamine production from several mutants grown on glutamine fermentation medium.

Strains	GA(μg/ml)	GM(μg/ml)
C. glutamicum ATCC 13058	15.2	16.5
C. glutamicum KA 8	26.7	22.8
C. glutamicum KA 6	26.7	24.6
C. glutamicum KA 5	27.6	25.5
C. glutamicum KT	31.3	52.7
C. glutamicum KH	27.8	26.4

*abbreviation
GA : glutamate glumata
GM : glutamine

Table 6. Feedback inhibition of GS from *C. glutamicum* ATCC 13058

Compounds (8mM)	Relative activity (%)
None	100
ADP	42.2
Histidine	53.2
Glycine	60.9
Alanine	62.9
Serine	81.6
Tryptophan	82.3
GMP	92.4

Glutamate and glutamine production

The amount of glutamate and glutamine production from parental strain, arg⁻, his⁻ and trp⁻ auxotrophs were measured at the same growth condition as well as the same cell density in glutamine fermentation media. Investigating with parental strain and other auxotrophs, Table 5 showed that the amounts of glutamate and glutamine of all the auxotrophs were higher levels more than those of parental strain. This result indicated that the arginine auxotrophs had blocked in branch pathway accumulated glutamate and glutamine in the culture media.

Specially, the mutants, trp⁻ and his⁻ auxotrophs showed remarkably higher levels of glutamate and glutamine production. There results are assumed that the repression of GS synthesis by tryptophan might be derepressed by the tryptophan auxotroph strain, so that the glutamine production of main pathway is increased to be accumulated in the media.

Compared with Table 2, 3 and 4, it is supposed that higher levels of GDH synthesis of mutants in nitrogen excess condition promoted the accumulation of glutamate and glutamine in fermenting medium.

Feedback inhibition of GS

After dialysis of cell-free extract with 0.15mM Tris acetate buffer, reaction was carried out with the addition of several amino acids, ADP, GMP to examine feedback inhibition with 8mM concentration. As shown in Table 6, inhibition of GS activity by ADP suggested that GS is regulated by energy

Table 7. Enzyme levels of culture media grown with various amino acids as nitrogen source.

Compounds	Relative activities (%)		
	GDH	GS	GOGAT
None	100	100	100
Tryptophan	66.7	60.3	37.3
Proline	85.5	89.3	6.5
Glycine	65.8	82.1	26.7
Serine	80.3	60.1	69.7
Alanine	100.6	88.8	67.7
Tyrosine	83.9	82.2	64.6

Each amino acid is added at 250 $\mu g/ml$ final concentration.

charge as in *Micrococcus glutamicus*[12]. The results with amino acids implied that a system of feedback inhibition were effective in this microorganism, and this is notable because some amino acids have been reported to inhibit GS remarkably in many organisms. Histidine among these amino acids was the highest inhibitor for GS activities. It is clear that tryptophan inhibited similarily the GS activity compared with *E. coli*. Alanine and glycine was also less effectively inhibited as in *E. coli*.[13] Therefore, it is assumed that feed-back inhibition of GS in *C. glutamicum* was similar tendency with *E. coli* [11,12].

Metabolic repression by amino acid sources

To determine if GS, GDH, and GOGAT synthesis is subjected to be controlled by repression with amino acids, cells were grown aerobically in minimal salts (M2) media containing glucose as a carbon source, supplemented with different nitrogen sources. As shown in Table 7, the inhibition of GS and GOGAT synthesis is found in the cell free extracts cultured on the media containing nitrogen sources such as tryptophan, proline, glycine, serine, alanine and tyrosine. Amino acids products which inhibit GS were added to the medium to determine if they might also be involve in repression of GS, GDH, and GOGAT synthesis. Among several amino acids, specially tryptophan lower the GDH, GS, and GOGAT synthesis. It is evident from these studies that the GS, GDH, and GOGAT synthesis is markedly influenced by the nature and kinds of available nitrogen sources such as amino acids described above.

Reference

1. Shin, M.K.: Thesis of Master Degree, Korea Univ. (1984)
2. Katsumata, R., A. Ozaki, T. Oka and A. Furuya: *J. Bacteriol.*, **159,** 306 (1984)
3. Sung, H.J.: pH. D. Thesis, Kyoto University (1984)
4. Lowensten, J.H.: Methods in Enzymology, **13,** 55 (1969)
5. Legrain, C., S. Vissers, E. Dubois, M. Legrain and J.M. Wiame: *Eur. J. Biochem.*, **123,** 611 (1982)
6. Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall: *J. Biol. Chem.*, **193,** 265 (1951)
7. Stadtman, E.R., B.M. Shapiro, H.S. Kingdon and C.A. Woolfolk and J.S. Hubbard: *Adv. Enzymol. Regul.*, **6,** 257 (1968)
8. Tylor, Bonnie: *Ann. Rev. Biochem.*, **47,** 1127 (1978)
9. Adelberg, E.A., M. Mandel and G.C.C. Chen: *Biochem. Biophys. Res. Comm.*, **18,** 788 (1965)
10. Magasanik, B.: *Ann. Rev. Genet.*, **16,** 135 (1982)
11. Woolffolk, C.A. and E.R. Stadtman: *Arch. Biochem. Biophys.*, **118,** 736 (1967)
12. Tachiki, T., S. Wakisaka, H. Kumagai and T. Tochikura: *Agric. Biol. Chem.*, **45,** 1487 (1981)
13. Brenchley, J.E., C.A. Baker and L.G. Patil: *J. Bacteriol.*, **124,** 182 (1975)
14. Miller, J.H.: Experiments in Molecular Genetics. Cold Spring Harbor Lab., 125 (1972)