[J. Ferment. Technol., Vol. 63, No. 1, 5-10. 1985]

Ammonia Assimilation by Glutamine Synthetase/Glutamate Synthase System in *Brevibacterium flavum*

Ha-Chin Sung, Makoto Takahashi, Hisanori Tamaki, Takashi Tachiki, Hidehiko Kumagai, and Tatsurokuro Tochikura

Department of Food Science and Technology, Kyoto University, Kyoto 606, Japan

The formation of glutamine synthetase (GS) and glutamate synthase (GOGAT) in *Brevibacterium flavum* (ATCC 14067) was repressed by ammonia in the culture medium. Glutamate dehydrogenase (GDH)-defective mutants could grow in ammonia medium but glutamate dehydrogenase (GDH)-glutamine synthetase (GS) or glutamate dehydrogenase (GDH)-glutamate synthase (GOGAT) double-defective mutants could not. Glutamine synthetase (GS) and glutamate synthase (GOGAT) were formed constitutively in glutamate dehydrogenase (GDH)-defective mutants which grew and accumulated a small amount of glutamic acid in the glutamate fermentation medium containing high concentration of ammonia as the main nitrogen source. When the cell-free extract of a glutamate dehydrogenase (GDH)-defective mutant was incubated with a mixture containing ammonium chloride, citrate and other substances for the glutamine synthetase (GS) and glutamate synthase (GOGAT) reactions, significant amount of glutamate was synthesized. These results suggested that glutamine synthetase (GS)/glutamate synthase (GOGAT) system could function for the assimilation of ammonia into glutamate in *B. flavum*.

It is now generally accepted that the glutamine synthetase (GS)/glutamate synthase (GOGAT) system is a major route of uptake of ammonia into the glutamate in bacteria in the presence of limited ammonia, and glutamate dehydrogenase (GDH) can function only when sufficient ammonia is available.[1-3] We are interested in the function of the GS/GOGAT system in *Brevibacterium flavum*, one of the most powerful glutamate-producing bacterium, because it has a high level of GDH which plays an important role in glutamate production, and because the function of the GS/GOGAT system in this organism is not sufficiently understood.

Previously we found that GS and GOGAT were present in *B. flavum* and that their activities varied with the nitrogen sources in the culture medium.[4] We also reported on the properties of GS and GOGAT from this organism.[5,6] Our findings suggested that the GS/GOGAT system might participate in the assimilation of ammonia at low concentration, even though there is some difference in properties between GOGATs from *B. flavum* and other sources.[5,6] If this system operates in *B. flavum*, it would be valuable in field of applied microbiology as well as in general microbiology.

In this study, we investigated the effect of nitrogen sources in the culture medium especially on the formation of GS, GOGAT and GDH and obtained results indicating that the synthesis of GS and GOGAT was repressed by ammonia whereas that of GDH was not. We also found that mutants defective in GDH could grow in a medium containing ammonia as the sole nitrogen source but GDH-GS and GDH-GOGAT double-defective mutants could not. These results suggested that the GS/GOGAT system could function in ammonia assimilation in *B. flavum*.

Materials and Methods

Bacteria, media and culture conditions *Brevibacterium flavum* (ATCC 14067) was used. To

prepare cell-free extracts, cells were cultured in 500 ml of media in a 2-*l* Sakaguchi flask with shaking (110 strokes per min) for 24–48 h at 30°C. For mutagenesis and investigation of growth characteristics, cells were cultured in 5 ml of medium in a 16-mm diameter test-tube with shaking or on 1.5% agar plate for 24-48 h at 30°C. Medium 1 contained 5 g of glucose, 5 g of peptone and 3 g of yeast extract per liter of water, pH 7.0. Medium 2 was composed of 10 g of glucose, 0.2 g of yeast extract, 0.05 g of KH_2PO_4, 0.05 g of K_2HPO_4 and 0.03 g of $MgSO_4 \cdot 7H_2O$ per liter of water, pH 7.0. Medium 3 was a synthetic medium containing 50 g of glucose, 1.5 g of KH_2PO_4, 0.6 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $FeSO_4$, 0.01 g of $MnSO_4$, 1.0 g of casamino acids (vitamin free), 0.01 mg of folic acid, 0.01 mg of biotin, 0.2 mg of riboflavin, 0.2 mg of thiamine-HCl, 0.2 mg of *p*-aminobenzoic acid, 0.1 mg of pyridoxine-HCl, 0.1 mg of calcium panthothenate, 0.1 mg of nicotinic acid, 0.02 mg of pyridoxal-HCl, 0.1 mg of adenine, 0.1 mg of guanine, 0.1 mg of uracil and 0.1 mg of xanthine per liter of water, pH 7.0. Various nitrogen sources were added to medium 2 or 3. The glutamate fermentation medium contained 36 g of glucose, 1 g of KH_2PO_4, 1 g of $MgSO_4 \cdot 7H_2O$, 10 mg of $FeSO_4 \cdot 7H_2O$, 10 mg of $MnSO_4 \cdot 4H_2O$, 100 μg of thiamine-HCl, 2 μg of biotin, 6 ml of "Mieki" (acid hydrolysate of soybean protein) and 15 g of ammonium sulfate per liter of water, pH 7.0. Cultivation was carried out in 20 ml of the medium with 1 g of $CaCO_3$ in a 500-ml Sakaguchi flask with shaking at 30°C.

Isolation of mutants GDH-defective mutants were isolated after mutagenesis with ethylmethanesulfonate, basically according to the method of Necasek *et al.*[7] *B. flavum* cells cultured overnight in liquid medium 1 were harvested by centrifugation (12,000 × *g*), washed twice with 1/15 M potassium phosphate buffer (pH 7.0) and resuspended in 3 ml of the buffer (OD at 610 nm : 1.0). Ethylmethanesulfonate was added to a final concentration of 0.05 M. After shaking for 20 h at 30°C, the cells were collected and resuspended in 0.85% saline. The cell suspension was spread on agar plates of medium 1 and incubated for 48 h at 30°C. Colonies appeared on the plates were replicated on agar plates of medium 2 containing 1 mM and 300 mM ammonium chloride. GDH-defective mutants were expected to grow on the medium with 1 mM ammonium chloride but not on that with 300 mM ammonium chloride. The growth characteristics of the isolated strains were further confirmed by their response to nitrogen sources in liquid medium 3. Deficiency of GDH was confirmed with cell-free extracts by determination of GDH activity and by double immunodifussion analysis with GDH antibody.

GDH-GS and GDH-GOGAT double-defective mutants were derived from GDH-defective mutant 8D. After mutagenesis and replica-plating in the same manner as above, cells which could grow on the agar plate of medium 3 supplemented with 30 mM sodium glutamate plus 0.18 mM glutamine but neither with 50 mM ammonium chloride nor with 30 mM sodium glutamate were isolated as GDH-GS defective mutants. Likewise, cells which could grow on the medium with 30 mM sodium glutamate but not with 50 mM ammonium chloride were picked up as GDH-GOGAT defective mutants. Their growth characteristics and immunological protein detection were confirmed in the same manner as those of the GDH defective mutant.

Enzyme assay Preparation of cell-free extracts and determination of the enzyme activities were carried out as described previously.[4] One unit of GOGAT and GDH activity was defined as the amount which oxidizes 1 μmol of NADPH per min, and that of GS as the amount which required for the synthesis of 1 μmol of γ-glutamylhydroxamate per min.

Immunological test Homogeneous GDH and GS and their antibodies were prepared as described previously.[6] Double immunodifussion analysis was carried out according to the method of Ouchterlony.[8]

Assays Protein was determined by the method of Lowry *et al.* with egg albumin as the standard.[9] Amino acids in the culture broth or in the reaction mixture were determined by paper chromatography.[10] α-Keto acids in the culture broth was determined by the method of Friedemann and Haugen[11] with α-ketoglutarate (α-KGA) as the standard. Bacterial growth in liquid medium was expressed as turbidity at 610 nm.

Chemicals Pyruvate kinase was from Sigma Chemical Co. All other reagents were the highest grade commercial products. All amino acids were the L-isomers.

Results and Discussion

Effect of nitrogen sources on the enzyme level We previously found that *B. flavum* cells grown on medium 2 with 1.12% (60 mM) sodium glutamate as a major nitrogen source contained high levels of GS and GOGAT.[4] When peptone or yeast extract was substituted for sodium glutamate, the activity of GOGAT decreased but there was no significant change in that of GS, even though GS activity was reduced in other bacteria grown in nutrient broth.[2,12] The activities of both GS and GOGAT decreased in the culture medium containing ammonia. GDH level did not vary with

nitrogen source. However, the findings with medium 2 do not allow discussion of the function of the GS/GOGAT system in *B. flavum*, because cell growth in this medium was very poor compared with that in the glutamate fermentation medium. On the other hand, during the investigation of the cultural conditions for GOGAT production,[5] we found that the extent of cell growth in medium 2 supplemented with 0.28% yeast extract and 0.56% (30 mM) sodium glutamate reached almost the same level as that in the glutamate fermentation medium. Moreover the level of GOGAT was further increased by addition of EDTA-Fe to this yeast extract and glutamate medium (Table 1, Exp. A).

Experiment B in Table 1 shows that the activities of both GS and GOGAT decreased with increase of ammonia concentration in the medium, as has been observed in other bacteria possessing a functional GS/GOGAT system under ammonia-limited conditions.[2]

Ammonia assimilation by mutants defective in the enzymes involved in glutamate biosynthesis Table 2 demonstrates that GDH-defective mutants (Fig. 1) could assimilate ammonia as the sole nitrogen source, even if the growth was not so good as the wild-type. It also indicates that the levels of GS and GOGAT in the mutants, especially grown on ammonium chloride, were higher than those in the wild-type strain. As the formation of GS was repressed by ammonia and that of GOGAT by yeast extract and ammonia in wild-type strain grown on medium 2,[4] this finding suggested some change in the regulation system for the synthesis of GS and GOGAT, respectively in the mutants. Table 3 indicates growth of the mutants in the media containing different amounts of ammonia as the sole nitrogen source and shows that the GDH-defective mutant 8D, as well as other strains (data not shown), grew similarly to the wild-type strain in the media with lower concentration of ammonia (less than 1 mM, 14 ppm nitrogen). The growth decreased with in-

Table 1. Effect of nitrogen sources and EDTA-Fe in medium on the levels of GS, GOGAT and GDH.

Supplement		Cell growth	GS	GOGAT	GDH
		(OD at 610 nm)	(units/mg protein)		
Exp. A					
0.28% Yeast extract		2.8	3.76	0.004	0.90
0.56% (30 mM) Sodium glutamate		1.8	4.90	0.035	0.89
0.28% Yeast extract and 0.56% sodium glutamate		16.4	3.94	0.029	1.41
0.56% Sodium glutamate and 0.01% EDTA-Fe		1.5	4.50	0.027	0.70
0.28% Yeast extract, 0.56% sodium glutamate and 0.01% EDTA-Fe		17.8	3.56	0.065	1.32
Exp. B					
Ammonium chloride	0 mM	17.8	2.42	0.060	0.91
	1 mM	16.2	2.02	0.047	0.91
	5 mM	16.4	3.30	0.017	1.14
	10 mM	18.4	0.35	0.001	1.02
	30 mM	17.0	0.40	0.001	0.93

Exp. A: Medium 2 was supplemented as indicated in the Table.
Exp. B: Various amounts of ammonium chloride were added to medium 2 supplemented with 0.28% yeast extract, 0.56% sodium glutamate and 0.01 % EDTA-Fe.

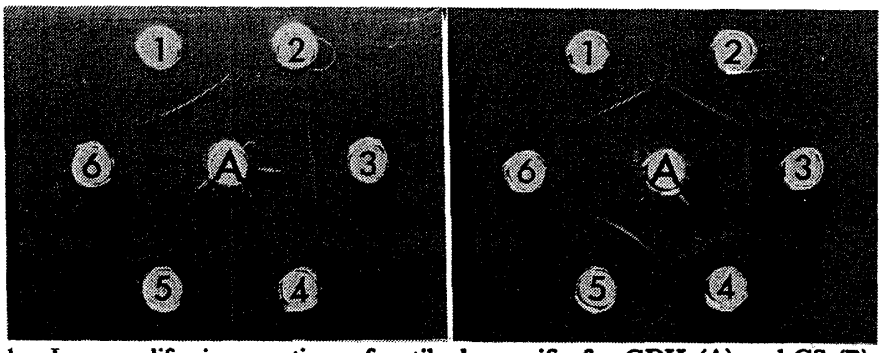

Fig. 1. Immunodifussion reactions of antibody specific for GDH (A) and GS (B) with cell-free extracts of the wild-type strain and mutants of *B. flavum*.
(A) GDH antibody (50 μg) was placed in center well. Well 1 contained 3 μg of GDH. Wells 2, 3, 4, 5 and 6 contained 35 μg, 38 μg, 30 μg, 40 μg and 37 μg of protein of cell-free extracts of 8D (GDH-defective), wild-type strain, 059 (GDH-GOGAT double-defective), 89 (GDH-defective) and S13 (GDH-GS double-defective), respectively. (B) GS antibody (50 μg) was placed in center well. Well 1 contained 5 μg of GS. Wells 2, 3, 4, 5 and 6 contained 38 μg, 28 μg, 36 μg, 30 μg and 35 μg of protein of cell-free extracts of wild-type strain, 8D (GDH defective), 059 (GDH-GOGAT double-defective), 89 (GDH-defective) and S13 (GDH-GS double-defective), respectively.

crease in ammonia concentration but was observed even in the maximum, 300 mM ammonia medium. The mutant 8D also grew in the glutamate fermentation medium[18] which contains a high concentration of ammonia as a major nitrogen source, and accumulated significant amount of glutamate and α-keto acids (Fig. 2). Enzyme activity of the mutant grown on glutamate fermentation medium was almost the same as in Table 2 (data not shown). Table 3 also shows that neither the GDH-GS double-defective mutant S13 (see Fig. 1) nor the GDH-GOGAT double-defective mutant 059 which contained GOGAT at 10% of the level of the wild-type strain, could grow at any concentration of ammonia.

The finding that the GDH-defective mutants could grow in the medium with high concentration of ammonia was unexpected, because in the wild-type strain the formation of GS and GOGAT was repressed under this condition (Table 1). The reason for this assimilation of ammonia should be discussed in relation to the change of the regulation system for the enzyme synthesis. That aside, the results of this study suggested that GS and GOGAT played a role in

Table 2. Growth and enzyme activities in the wild-type strain and GDH-defective mutants of *B. flavum* grown on ammonia or yeast extract as a major nitrogen source.

Strain	Nitrogen source	Cell growth	GS	GOGAT	GDH
		(OD at 610 nm)	(units/mg protein)		
Wild-type	Ammonium chloride	1.50	0.59	0.006	1.17
	Yeast extract	4.80	2.12	0.011	1.08
8D (GDH-defective)	Ammonium chloride	0.32	1.70	0.051	0
	Yeast extract	0.97	2.08	0.058	0
8F (GDH-defective)	Ammonium chloride	0.27	2.20	0.090	0
	Yeast extract	0.95	3.01	0.029	0
89 (GDH-defective)	Ammonium chloride	0.30	1.30	0.079	0
	Yeast extract	0.87	1.28	0.068	0

The organisms were grown at 30°C for 24 h on medium 2 supplemented with ammonium chloride (5 mM) or yeast extract (0.48%) as the sole nitrogen source.

Table 3. Growth response to ammonia concentration of the wild-type strain and mutants defective in one or more enzymes in glutamate biosynthesis in *B. flavum*.

Ammonium chloride (mM)	Cell growth (OD at 610 nm)			
	Wild	8D*	S13*	059*
0	0.03	0.02	0.02	0.01
0.5	0.33	0.32	0.01	0.01
1	0.45	0.40	0.02	0.01
5	1.00	0.40	0.02	0.02
10	1.10	0.40	0.02	0.02
100	0.90	0.35	0.01	0.01
300	0.30	0.10	0.01	0.01

*8D : GDH-defective
S13 : GDH-GS double-defective
059 : GDH-GOGAT double-defective
The organisms were grown at 30°C for 24 h on medium 2 supplemented with various concentration of ammonium chloride as the sole nitrogen source.

ammonia assimilation in *B. flavum* under the ammonia-limited condition and that both enzymes were indispensable for glutamate formation in GDH-defective mutants, as

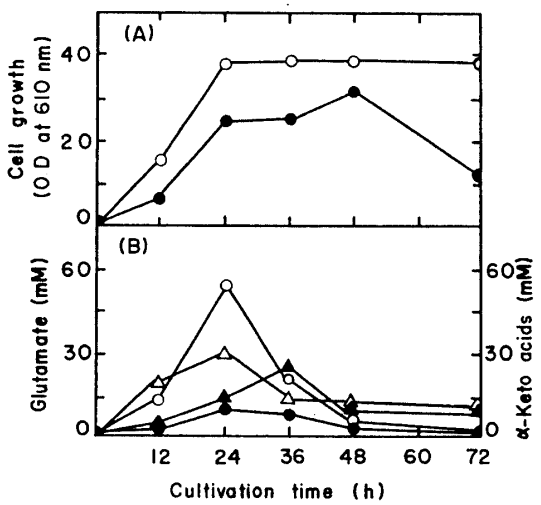

Fig. 2. Growth of and formation of glutamate and α-keto acids by the wild-type strain and GDH-defective mutant 8D of *B. flavum* in the glutamate fermentation medium.
(A) Cell growth. Symbols: —○—, wild-type strain; —●—, 8D (GDH-defective).
(B) Formation of glutamate and α-keto acids. Symbols: —○—, glutamate by wild-type strain; —●—, glutamate by 8D (GDH-defective); —△—, α-KGA by wild-type strain; —▲—, α-KGA by 8D (GDH-defective).

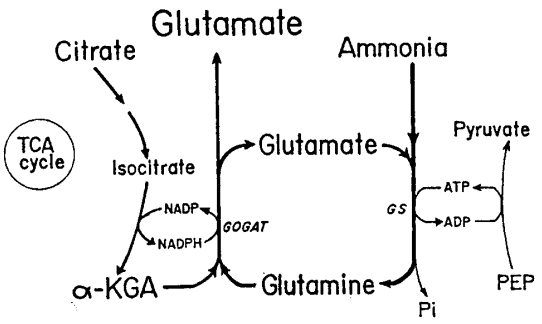

Fig. 3. Synthesis of glutamate by the GS/GOGAT system.

has been reported with GDH-defective mutants of *Escherichia coli*[14] and *Bacillus megaterium*.[15] These indications were strongly supported by the demonstration of the consecutive reactions of GS and GOGAT (illustrated in Fig. 3) in the cell-free extracts of the GDH-defective mutant (Fig. 4). During the reactions, NADPH was regenerated by the TCA cycle for the synthesis

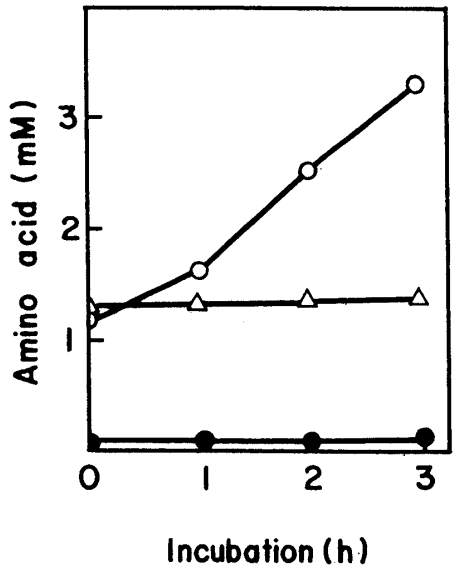

Fig. 4. Formation of glutamate from ammonia and citrate by cell-free extracts of GDH-defective mutant 8D.
The reaction mixture contained 25 mM ammonium chloride, 7 mM citrate, 1.3 mM glutamate, 1 mM ATP, 0.1 mM NADPH, 30 mM $MgCl_2$, 1 mM $CoCl_2$, 13 mM phosphoenolpyruvate, 25 units/ml of pyruvate kinase, 50 mM Tris-HCl buffer (pH 7.5) and 3.0 mg/ml of protein of cell-free extracts. Incubation was carried out at 30°C.
Symbols: —○—, glutamate; —●—, glutamine; —△—, glutamate in the control mixture (minus pyruvate kinase-phosphoenolpyruvate system).

of α-KGA from citrate, and ATP by the pyruvate kinase-phosphoenol pyruvate system. Addition of a small amount of glutamate was necessary as the initial substrate for GS to start the reactions.

The nonrepressive formation of GS and GOGAT in the wild-type strain under the ammonia limited-condition and the growth characteristics of the mutants defective in GDH, GS and GOGAT revealed the important role of GS/GOGAT system in ammonia assimilation by *B. flavum*. However, it should be noted that most of the present results were obtained with mutants which synthesized GS and GOGAT constitutively. Deeper insight might be afforded by analyzing the change in pool of amino acids, especially glutamine and glutamate, with ammonia concentration[16,17] and the growth characteristics of mutants impaired only in the GS/GOGAT system.

Acknowledgments

We express our thanks to the Ajinomoto Co., Inc., for the kind gift of some chemicals.

References

1) Brown, C. M., MacDonald-Brown, D. S. Meers, J. L.: *Adv. Microbiol. Physiol.*, **11**, 1 (1974).
2) Tyler, B.: *Ann. Rev. Biochem.*, **47**, 1127 (1978).
3) Magasanik, B.: *Ann. Rev. Genet.*, **16**, 135 (1982).
4) Tochikura, T., Sung, H. C., Tachiki, T., Kumagai, H.: *Agric. Biol. Chem.*, **48**, 2149 (1984).
5) Sung, H. C., Tachiki, T., Kumagai, H., Tochikura, T.: *J. Ferment. Technol.*, **62**, 371 (1984).
6) Sung, H. C., Tachiki, T., Kumagai, H., Tochikura, T.: *J. Ferment. Technol.*, **62**, 569 (1984).
7) Necasek, J., Pikalek, P., Drobink, J.: *Mutation Res.*, **4**, 409 (1967).
8) Ouchterlony, O.: *Handbook of Immunodifussion and Immunoelectrophoresis*, p. 1, Ann Arbor Science Publishers, Ann Arbor, Mich. (1968).
9) Lowry, O. H., Rosebrough, N. T., Farr, A. L., Randall, R. J.: *J. Biol. Chem.*, **193**, 265 (1951).
10) Katagiri, H., Soda, K., Tochikura, T.: *Nippon Nogeikagaku Kaishi*, **34**, 814 (1960).
11) Friedemann, T. E., Haugen, G.: *J. Biol. Chem.*, **147**, 415 (1943).
12) Brencheley, J. E., Baker, C. A., Patil, L. B.: *J. Bacteriol.*, **124**, 172 (1975).
13) Ozaki, H., Shiio, I.: *Agric. Biol. Chem.*, **47**, 1569 (1983).
14) Berberich, M. A.: *Biochem. Biophys. Res. Commun.*, **47**, 1498 (1972).
15) Elmerich, C., Aubert, J. P.: *Biochem. Biophys. Res. Commun.*, **42**, 371 (1971).
16) Senior, P. J.: *J. Bacteriol.*, **123**, 407 (1975).
17) Despande, K. L., Katze, J. R., Kane, J. F.: *J. Bacteriol.*, **145**, 768 (1981).

(Received July 18, 1984)