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Isolation and Characterization of glutamate dehydrogenase defective mutant of *Brevibacterium flavum*

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*Brevibacterium flavum*의 glutamate dehydrogenase 결핍돌연변이주의 분리 및 특성

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ABSTRACT: In order to understand the regulation of glutamate dehydrogenase(GDH) synthesis in *Brevibacterium flavum*, we have isolated a mutant lacking NADP-linked GDH activity by ethylmethane sulfonate treatment. The gdh⁻ mutant was grown on the minimal plate with 1mM ammonium chloride and not that with 300mM ammonium chloride. The cell-free extracts from gdh⁻ mutant and prototroph were also examined with glutamine synthetase(GS) and glutamate synthase(GOGAT) production by nitrogen sources. The growth of gdh⁻ mutant in presence of 20mM ammonium chloride means that GOGAT synthesis is sufficient to allow growth in this condition. GS production of gdh⁻ mutant as well as parental strain was induced by 1mM urea and ammonium tartrate, but it was repressed by higher concentration of ammonia, and also induced by 20mM to 50mM glutamate as a substrate. It was special attention that GOGAT synthesis from gdh⁻ strain was more repressed by higher concentration of ammonia than prototroph as described in *E. coli* system.

KEY WORDS: Characterization of gdh⁻ mutant, nitrogen assimilation, regulation of GDH, GS and GOGAT synthesis.

Until the discovery of a glutamate synthase (GOGAT) activity in microorganisms (Meers *et al.*, 1970; Tempest *et al.*, 1970), glutamate dehydrogenase (GDH) was thought to be the only enzyme responsible for glutamate synthesis. Studies with mutants of *Klebsiella aerogenes* (Brenchley *et al.*, 1973; Brenchley and Magasanik, 1974), *Escherichia coli* (Berberich, 1972; Pahel *et al.*, 1978), and *Salmonella typhimurium* (Brenchley *et al.*, 1975; Dendinger *et al.*, 1980) demonstrated that either GDH or GOGAT synthesis is sufficient to allow growth in media with excess ammonia. However, when cells are grown with limiting ammonia or with certain nitrogen sources, only the GOGAT reaction is essencial. The loss of both enzyme activities causes a glutamate requirement independent of ammonia supply. The function of GDH in *S. typhimurium* is interesting, because this enzyme activity did not vary during the cell growth with different nitrogen sources (Brenchley *et al.*, 1975; Rosenfeld *et al.*, 1982). In contrast, in *K. aerogenes*, GDH activity is markedly decreased when the cells were grown with a limiting nitrogen source (Brenchley *et al.*, 1973). In order to understand the regulation of GDH synthesis in *S. typhimurium*, they had isolated a mutant lacking this enzyme activity. Recently, Dendinger *et al.* (1980) described the isolation of mutants with a

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heat-labile GDH enzyme. Specially, *Brevibacterium flavum* produces large amount of glutamate by GDH in presence of ammonia. We have already examined purfication and properties of GDH and GS from Coryneform bacteria in the same group of microorganisms as classification status.

In this paper, in order to the regulation of GDH synthesis in *B. flavum*, we have isolated mutant, gdh$^-$ strain, lacking this enzyme activity. However, this gdh$^-$ mutant was able to grow on the medium contained ammonium chloride because it produced GOGAT synthesis. We will describe here the characteristics of enzymatic regulation in gdh$^-$ mutant.

MATERIALS AND METHODS

Bacterial Strains: The strains of bacteria used in this experiments are shown in Table 1.

Isolation of GDH Defective Mutant

B. flavum cells cultured in the complete broth at 30 °C for 16 hours (4.3×10^6 cells/m*l*). The cultures were then centrifuged (12,000 × g), washed twice with 1/15M potassium phosphate buffer (pH 7.0) and resuspended in 3 m*l* of the buffer. The optical density of the culture was measured at 610 nm using a spectrophotometer (O.D. at 610 nm: 1.0). Exposure to the culture with 12.5 mM ethylmethane sulfonate (EMS) was carried out with shaking at 37 °C for 20 hours, the cells were collected and resuspended in 0.85% saline. The cell suspension was spreaded on the complete plate and incubated for 48 hours at 30 °C. Colonies appearing on the plates were replicated on the complete plates. Colonies were selected on the minimal plates with 1 mM and 300 mM ammonium chloride since gdh$^-$ mutants were expected to grow on the minimal plate with 1 mM ammonium chloride but not on that with 300 mM ammonium chloride.

Table 1. *List of Brevibacterium flavum strains and their characteristics.*

Strain	Relevant genotype	source
B. flavum ATCC 14067	prototroph	ATCC*
B. flavum SUB 12	gdh$^-$	This laboratory

*American Type Culture Collection

Media and cell cultivation: The media and culture method were as described by Sung (1984). Cultures were incubated for 24 hours at 30 °C with shaking in 200m*l* Erlenmeyer flasks containing 20 m*l* complete broth and 0.4 m*l* of inoculum. The inoculum was 5 m*l* of a nutrient broth culture grown overnight. The complete medium contained 5 g glucose, 5 g peptone, 3 g yeast extract, 20 g agar per liter of distilled water. In order to investigate the effect of nitrogen sources on glutamate dehydrogenase, glutamine synthetase, and glutamate synthase production, the minimal media were used with different concentration of ammonium tartrate, urea, glutamate, and glutamine. Minimal medium contained 1.07g NH_4Cl(20mM), 1g K_2HPO_4, 50mg NaCl, 20g dextrose, 0.04g $MgSO_4 \cdot 7H_2O$,, 0.2mg $FeSO_4 \cdot 7H_2O$, 0.2mg $MnSO_4 \cdot 6H_2O$, 50 μg biotin, and 200 μg thiamine·HCl per liter of distilled water.

Preparation of Cell Extract: Cells of both strains were cultured in minimal broth with different concentrations of nitrogen sources instead of ammonium chloride for 24 hours at 30 °C. Cells were centrifuged, the supernatant was discarded and the cell pellet was harvested and sonicated with sonic dismembrator (Model 300, Fisher) to be broken. The broken cells were centrifuged at 10,000 rpm for 10 minutes at 0 °C and the supernatant was used as the cell-free extract.

Enzyme Assays: All cell-free extracts were made in 100mM Tris-HCl buffer, pH 7.0. Glutamate dehydrogenase (GDH) of cell-free extract was assayed according to the modified procedure of Kimura *et al.*(1977). The reduction of NADPH was measured by the change in E_{340} in a recording spectrophotometer. The assay mixture contained 50mM NH_4Cl, 7.0mM α-ketoglutaric acid, 0.15 mM NADPH, 100mM potassium phosphate buffer (pH 7.0) and the enzyme protein to be a final volume of 2.5 m*l*. The initial rate of reaction was determined in the presence and absence of ammonium chloride at 37 °C. Specific activities are expressed as 1 μmole NADPH oxidization per minute per mg of protein.

Glutamine synthetase(GS) assay was carried

out by measuring γ-glutamyltransferase activity by Shapiro and Stadtman (1967). The assay mixture composed of 3.5 mM glutamine, 3.5mM α-ketoglutaric acid, 0.15mM NADPH, 100mM potassium phosphate buffer (pH 7.0) and adjusted to pH 7.57 with 2M triethanolamine, to be 23m*l* with distilled water, added with 0.4mM $MnCl_2$. The 1m*l* of assay reagent was reacted with 1m*l* of enzyme solution and incubated at 37 °C for 30minutes, added with 1m*l* of stop mixture to stop react. The reaction mixture was centrifuged at 12,000rpm for 3 minutes. Precipitate was removed and absorbancy was measured at 540nm. One unit of the enzyme was defined as the amount required to catalyze the synthesis of 1 μ mole of γ-glutamylhydroxamate per minute at 30 °C and one specific activity unit of the enzyme was defined as 1 unit/mg protein.

Glutamate synthase (GOGAT) assay was carried out by the method of Miller *et al.*(1972). Assay mixture contained 3.5mM glutamine, 3.5mM α-ketogutaric acid, 0.15mM NADPH, 100mM potassium phosphate buffer (pH 7.0). The assay mixture, 2.4m*l*, was reacted with 0.1m*l* of enzyme solution, and was measured initial rate of NADPH oxidation. One unit of enzyme activity was defined as the amount which oxidizes 1 μ mole of NADPH per minute.

Protein Determination: The amount of soluble protein in cell-free extracts were determined by the method of Lowry *et al.* (1951).

RESULTS AND DISCUSSION

Isolation of Glutamate Dehydrogenase Defective Mutant

Glutamate dehydrogenase defective mutant from *B. flavum* ATCC 14067 was isolated by mutagenesis with ethylmethane sulfonate (EMS) according to the method of Necasek *et al.* (1967). EMS was added directly to 16 hours cultures (4.3×10^6 cells/m*l*) of *B. flavum* ATCC 14067 growing in the complete medium, at final concentration of 12.5 mM, 25 mM, and 50 mM EMS as shown in Table 2. The one disadvantage to the use of EMS as a mutagen is the high probability that every cell will be mutated at more than one site. Therefore, we chose the LD_{50} condition which is treated with 12.5 mM of EMS for the selection of gdh^- mutants. It was confirmed that this mutant was grown on the minimal plates with 1mM ammonium chloride but not that with 300mM ammonium chloride as shown in Table 3. GDH defective mutant was also confirmed with cell-free extract by determination of GDH activity and by double immunodiffusion analysis with GDH-antibody (Sung, 1984).

Table 2. *Survival as a function EMS concentration*

EMS (mM)	Survival (%)
0	100
12.5	46.90
25	0.07
50.0	0.02

Table 3. *Growth characteristics of gdh^- mutant on the minimal plate with ammonium chloride.*

Growth substrate \ Strain	gdh^- mutant	Prototroph
1mM NH_4Cl	+	+
300mM NH_4Cl	−	+

+ ; good growth, − ; no growth

Effect of Ammonium Chloride on the Growth of *Brevibacterium flavum* SUB12 (gdh^- mutant)

In order to determine the optimum concentration of ammonium chloride for the growth of *Brevibacterium flavum,* flasks contained minimal broth with ammonium chloride were inoculated with inoculum to be 0.02 with the initial optical density at 610nm. The cells were cultured at 30 °C on an rotary shaker at about 180 rev. per minute. Both strains of *B. flavum* ATCC 14067 and *B. flavum* SUB12 (gdh^- mutant) were grown in minimal medium contained the various concentrations of ammonium chloride as a sole nitrogen source as shown in Table 4. The cell growth was effected with the increasing concentrations of ammonium chloride. The optimum concentration of ammonium chloride in the minimal broth was 20mM for the cell growth. Specially, the growth of

Table 4. *The growth of B. flavum SUB12(gdh⁻ mutant) and its parent strain, ATCC14067, on the concentration of ammonium chloride*

NH_4Cl concentration (mM)	Growth of gdh⁻ mutant (O.D. at 610nm)	Growth of parental strain (O.D. at 610nm)
1	0.03	0.31
2	0.16	0.64
10	0.40	0.87
20	0.69	1.02
50	0.23	0.63
80	0.07	0,53
100	0.05	0.33

gdh⁻ mutant (SUB12) cells was very low in presence of ammonium chloride even if mutant strain lacks GDH. This growth of gdh⁻ mutant means that GOGAT synthesis is sufficient to allow growth in media with 20 mM ammonia showing agreement with other microorganisms (Berberich, 1972; Brenchley *et al.*, 1973, 1974, 1975; Pahel *et al.*, 1978). The loss of both enzyme activities, GDH and GOGAT, causes a glutamate requirement independent of ammonia supply. Actually this gdh⁻ mutant shows somewhat high activities of GOGAT in presence of ammonium tartrate or urea as shown in Figure 5.

Enzyme Presence of Intra- and Extracellular Distribution

The cells of parental strain, *B. flavum* ATCC 14067, and mutant strain, *B. flavum* SUB12 were cultured in minimal broth with 20mM ammonium chloride for 24 hours at 30 °C. The cells were centrifuged, the supernatant was used as extracellular enzyme solution. Sonically treated crude extract of the cells was used as intracellular enzyme solution. As shown in Table 5, the presence of glutamate dehydrogenase was distributed in intracellular, whereas glutamine synthetase was presented as intra- and extracellular enzyme in both strains.

Table 5. *Cellular distribution of glutamate dehydrogenase(GDH) and glutamine synthetase(GS)*

Strain	Cellular distribution	Enzyme	Specific activities (μM/min/mg protein)
ATCC-14067 (prototroph)	Intra-	GDH	8.7×10^{-1}
		GS	3.6×10^{-3}
	Extra-	GDH	0.0
		GS	4.0×10^{-4}
SUB 12 (gdh⁻ mutant)	Intra-	GDH	1.4×10^{-2}
		GS	4.0×10^{-3}
	Extra-	GDH	0.0
		GS	4.0×10^{-4}

Effect of Nitrogen Source on Glutamate Dehydrogenase Synthesis

B. flavum SUB12(gdh⁻ mutant) and *B. flavum* ATCC 14067 were grown under the conditions with ammonium tartrate, urea, L-glutamate, and L-glutamine as sole nitrogen sources with concentrations ranging from 5mM to 100mM. The cells were harvested and prepared cell extract according to the "Methods and materials". The specific activity of glutamate dehydrogenase was determined comparing with gdh⁻ mutant, *B. flavum* SUB12 and parental strain, *B. flavum* ATCC 14067. The results are shown in Figure 1. It was found that GDH activities of parental strain was increased to be the highest level at the concentrations of 20mM urea and ammonium tartrate, and they were decreased at 50mM or 100mM nitrogen concentrations. The growth of high concentra-

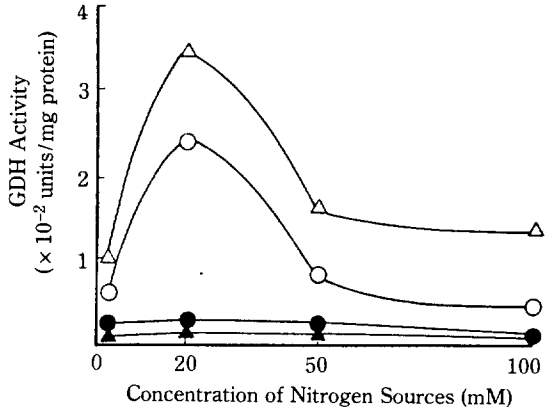

Fig. 1. *Glutamate dehydrogenase synthesis of B. flavum ATCC14067 and gdh⁻ mutant grown on various concentrations of urea and ammonium tartrate.*

△ ; urea in prototroph, ▲ ; urea in gdh⁻ mutant, ○ ; ammonium tartrate in parental strain, ● ; ammonium tartrate in gdh⁻ mutant.

tions of urea results in low activity of glutamate dehydrogenase. The possibility should be considered that urea repressed the synthesis of glutamate dehydrogenase. Pateman(1969) described that high concentration of urea repressed glutamate dehydrogenase in *Aspergillus nidulans*. In this respect, this result was shown in good agreement with that from *A. nidulans*.

Microorganisms that can utilize urea as a source of nitrogen produce urease, and consequently ammonia from urea. *Aspergillus* can grow on urea and U2 mutants (Scazzocchio and Darlington, 1967), which lack urease activity, are unable to utilize urea. A comparison of wild-type cells and a U2-4 mutant was made with respect to glutamate dehydrogenase activity after growth on media containing 100mM urea and 50mM nitrate, in an attempt to show whether urea itself or some metabolites produced from it affect glutamate dehydrogenase synthesis.

However the result in this experiment shows that 20mM urea induce glutamate dehydrogenase synthesis than ammonium tartrate. It was assumed that glutamate dehydrogenase synthesis of *B. flavum* requires some amount of ammonia from urea by urease.

Glutamate dehydrogenase synthesis from gdh$^-$ strain was very lower because this mutant lacks glutamate dehydrogenase activity with the addition of urea and ammonium tartrate.

B. flavum parental strain and mutant strain were also grown on concentration of glutamate or glutamine ranging from 5mM to 100mM. The cell extracts were assayed for glutamate dehydrogenase activity. The result is given in Figure 2. In the prototroph of *B. flavum*, glutamate dehydrogenase synthesis decreased dramatically with the increasing concentration of glutamate or glutamine. Specially, high concentration of glutamate repressed glutamate dehydrogenase synthesis because it is final product of enzyme. However, in mutant strain, glutamate dehydrogenase activity was very low with the addition of glutamate or glutamine, resulting from the cell extract of glutamate dehydrogenase defective mutant. It is supposed that mutant strain does not affected with product inhibition.

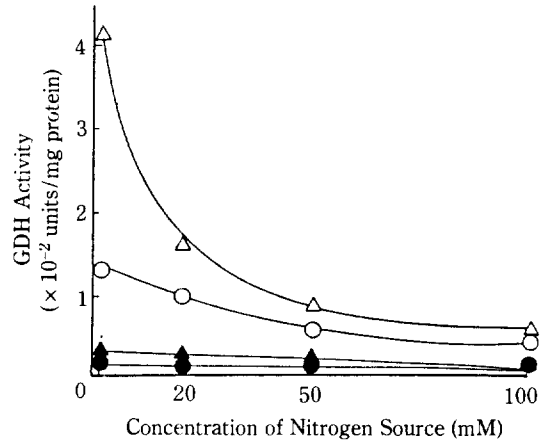

Fig. 2. *Glutamate dehydrogenase synthesis of B. flavum ATCC14067 and gdh$^-$ mutant grown on various concentrations of L-glutamate and L-glutamine.*
○ ; L-glutamate in parental strain, ● ; L-glutamate in gdh$^-$ mutant, △ ; L-glutamine in parental strain, ▲; L-glutamine in gdh$^-$ mutant.

Regulation of GS Synthesis by Nitrogen Sources

At the second step of nitrogen assimilation from α -ketoglutaric acid, GS synthesis was repressed by the addition of increasing concentration of urea and ammonium tartrate as shown in Figure 3. This result means that ammonia repres-

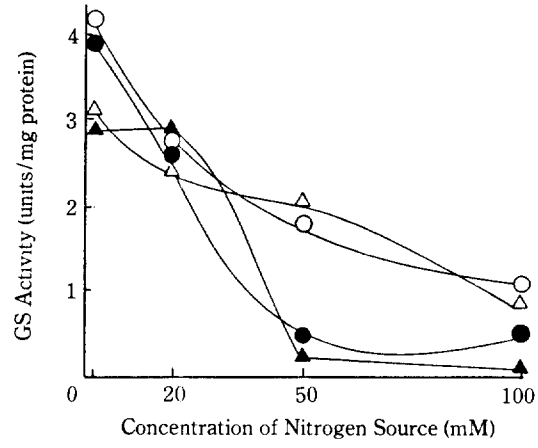

Fig. 3. *Glutamine synthetase synthesis of B. flavum ATCC14067 and gdh$^-$ mutant grown on various concentrations of urea and ammonium tartrate.*
△; urea in parental strain, ▲ ; urea in gdh$^-$ mutant, ○ ; ammonium tartrate in parental strain, ● ; ammonium tartrate in gdh$^-$ mutant.

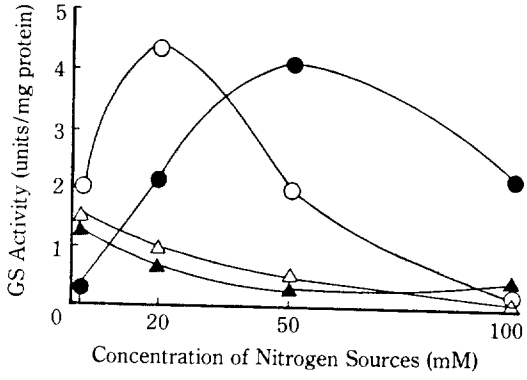

Fig. 4. *Glutamine synthetase synthesis of B. flavum ATCC14067 and gdh⁻ mutant grown on various concentrations of L-glutamate and L-glutamine.* ○; L-glutamate in parental strain, ●; L-glutamate in gdh⁻ mutant, △; L-glutamine in parental strain, ▲; L-glutamine in gdh⁻ mutant.

sed GS synthesis as reported in *E. coli* or *S. typhimurium* systems (Miller and Stadtman, 1967; Shapiro and Stadtman, 1967; Magasanik, 1982). Specially, in mutant strain, the GS production was more susceptible to ammonium concentration compared with prototroph. The effect of glutamate or glutamine on glutamine synthetase synthesis represents in Figure 4. In parental strain and mutant strain, GS production was remarkably induced to be the highest level at 20mM or 50mM glutamate, enzyme substrate, but decreased with higher concentration of glutamate, assuming substrate inhibition effect. However, the effect of glutamine on GS production showed the decrease of GS synthesis with the increasing concentrations of glutamine, suggesting that end product inhibition was represented in this system.

Regulation of GOGAT Synthesis by Nitrogen Sources

The cell extracts from both strains were determined glutamate synthase production. The results are shown in Figures 5 and 6. Glutamate synthase production from parental strain was higher than that of mutant strain.

Figure 5 showed that glutamate synthase production from both strain were maximum level at 1mM ammonium tartrate and urea, but it was rapidly decreased with increasing concentrations of nitrogen sources. This result means that ammonia

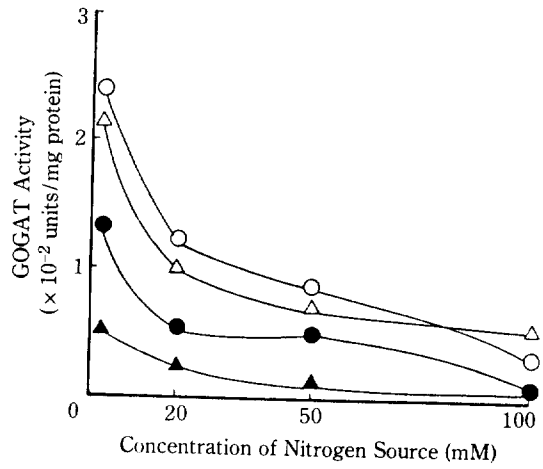

Fig. 5. *Glutamate synthase synthesis of B. flavum ATCC14067 and gdh⁻ mutant grown on various concentrations of urea and ammonium tartrate.* △; urea in parental strain, ▲; urea in gdh⁻ mutant, ○; ammonium tartrate in parental strain, ●; ammonium tartrate in gdh⁻ mutant.

repress glutamate synthase activity like *E. coli* system (Miller and Stadtman, 1972). It was special attention that glutamate synthase from mutant strain was lower than that of parental strain in presence of urea and ammonium tartrate.

Figure 6 represents the result that glutamate as enzymatic product induced glutamate synthase

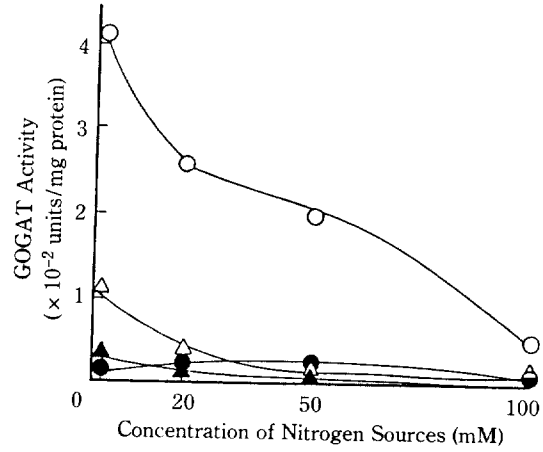

Fig. 6. *Glutamate synthase synthesis of B. flavum ATCC14067 and gdh⁻ mutant grown on various concentrations of L-glutamate and L-glutamine.* ○; L-glutamate in parental strain, ●; L-glutamate in gdh⁻ mutant, △; L-glutamine in parental strain, ▲; L-glutamine in gdh⁻ mutant.

production from both strains at lower concentration ranging from 1mM to 20mM. In case of this experiment, glutamate synthase production from gdh⁻ mutant strain was very low at any concentration of glutamate or glutamine. From this result, it seems that glutamate synthase production from mutant strain was also repressed by product inhibition, glutamate, and specially by glutamine.

적 요

*Brevibacterium flavum*의 glutamate dehydrogenase(GDH)의 합성조절을 규명하기 위하여 ethylmethane sulfonate를 처리하여 NADP-linked GDH 활성이 없는 gdh⁻ 돌연변이주를 분리하였다. gdh⁻ 돌연변이주는 1 mM ammonium chloride가 있는 최소배지에서 생장하였으나 300 mM ammonium chloride가 포함된 최소배지에서는 생장하지 못하였다. gdh⁻ mutant와 그의 prototroph의 GDH, glutamine synthetase(GS), glutamate synthase(GOGAT) 합성에 미치는 질소원의 영향을 검토하였다. 또한 gdh⁻ 돌연변이주가 20 mM ammonium chloride 존재하에서 가장 높은 생장을 보여 주었다. 이 결과는 이 mutant가 위 조건하에서 GOGAT를 합성하여 glutamate를 합성할 수 있기 때문에 생장이 가능한 것으로 해석된다. gdh⁻ mutant도 prototroph과 같이 GS 생성이 높았으며, 그것의 생성은 1 mM urea나 ammonium tartrate에 의해 유도되었으나 높은 농도의 ammonia에서는 억제되었다. 또한 기질인 20 mM내지 50 mM의 glutamate에 의하여도 GS 합성이 유도되었다. gdh⁻ mutant의 GOGAT 합성은 *E. coli* system과 같이 고농도의 ammonia에 의하여 억제됨을 관찰할 수 있었다.

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