Molecular Cloning and Characterization of a Large Subunit of *Salmonella typhimurium* Glutamate Synthase (GOGAT) Gene in *Escherichia coli*

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Two pathways of ammonium assimilation and glutamate biosynthesis have been identified in microorganisms. One pathway involves the NADP-linked glutamate dehydrogenase, which catalyzes the amination of 2-oxoglutarate to form glutamate. An alternative pathway involves the combined activities of glutamine synthetase, which aminates glutamate to form glutamine, and glutamate synthase, which transfers the amide group of glutamine to 2-oxoglutarate to yield two molecules of glutamate. We have cloned the large subunit of the glutamate synthase (GOGAT) from *Salmonella typhimurium* by screening the expression of GOGAT and complementing the gene in *E. coli* GOGAT large subunit-deficient mutants. Three positive clones (named pUC19C12, pUC19C13 and pUC19C15) contained identical *Sau*3AI fragments, as determined by restriction mapping and Southern hybridization, and expressed GOGAT efficiently and constitutively using its own promoter in the heterologous host. The coding region expressed in *Escherichia coli* was about 170 kDa on SDS-PAGE. This gene spans 4,732 bases, contains an open reading frame of 4,458 nucleotides, and encodes a mature protein of 1,486 amino acid residues (M_r = 166,208). The FMN-binding domain of GOGAT contains 12 glycine residues, and the 3Fe-4S cluster has 3 cysteine residues. The comparison of the translated amino acid sequence of the *Salmonella* GOGAT with sequences from other bacteria such as *Escherichia coli*, *Salmonella enterica*, *Shigella flexneri*, *Yersinia pestis*, *Vibrio vulnificus* and *Pseudomonas aeruginosa* shows sequence identity between 87 and 95%.

Keywords: glutamate synthase, large subunit, molecular cloning, *Salmonella typhimurium*

Glutamate is a central player in global nitrogen metabolism (Reitzer and Schneider, 2001; Reitzer, 2003); 75 to 90% of all cellular nitrogen is assimilated via glutamate. NH_4^+ is the preferred nitrogen source for *E. coli*, and it is assimilated into glutamate through two pathways. One of these pathways is the glutamate dehydrogenase (GDH) pathway, in which 2-oxoglutarate undergoes reductive

condensation with NH_4^+, yielding glutamate. The second pathway is the two-step glutamine synthetase (GS)-glutamate synthase (GOGAT) pathway, in which glutamine that is synthesized in the first step from one molecule of NH_4^+ and glutamate (in the presence of ATP) is involved in a reductive reaction with 2-oxoglutarate, which yields two molecules of glutamate (Nandineni *et al.*, 2004). GDH, GS, and GOGAT are encoded by the *gdhA*, *glnA*, and *gltBD* genes, respectively. The GDH pathway is functional for nitrogen assimilation in media containing ≥1 mM NH_4^+. In media with limiting NH_4^+ concentrations or

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with alternative poor nitrogen sources, nitrogen assimilation into glutamate is mediated through the GS-GOGAT pathway, whose regulation in turn is tied to the complex cascade of nitrogen regulation that is referred to as Ntr (Reitzer, 1996; Reitzer and Schneider, 2001; Reitzer, 2003). In NH_4^+-replete media, *glnA* is expressed only at basal levels; in addition, there is a reduction in the catalytic activity of GS resulting from the adenylylation of its homopolymeric subunits by the *glnE*-encoded adenylyltransferase. The residual activity of GS is then sufficient to meet the cell's anabolic requirement for glutamine for protein synthesis. On the other hand, in low-NH_4^+ medium or during growth on poor nitrogen sources, expression of the Ntr regulon (of which *glnA* is a member) is activated, and in addition GS is deadenylylated by GlnE; the vastly increased activity of GS is now able to catalyze sufficient glutamine synthesis to meet the cell's nitrogen assimilation requirement.

In *Salmonella typhimurium*, as in a number of other bacteria, there are two pathways for synthesis of glutamate: the glutamine synthetase/glutamate synthase (GS/GOGAT) cycle (Tempest *et al.*, 1970) and biosynthetic glutamate dehydrogenase (GDH). For each turn of the GS/GOGAT cycle, one molecule of ammonium/ammonia (NH_4^+) and one molecule of 2-oxoglutarate are assimilated into glutamate. Because GS has a high affinity for NH_4^+ (K_m < 0.2 mM) (Miller and Stadtman *et al.*, 1972) and the synthesis of glutamate is coupled to ATP hydrolysis, the GS/GOGAT pathway functions efficiently even at low NH_4^+ concentrations. By contrast, the GDH pathway functions efficiently only at high NH_4^+ concentrations, because GDH has a relatively low affinity for NH_4^+ (K_m > 1 mM) (Tempest *et al.*, 1970; Miller and Stadtman *et al.*, 1972). In *gltBD* mutant strains, which lack a functional GOGAT, glutamate synthesis depends upon GDH and can be limited at low external NH_4^+ concentrations (Csonka *et al.*, 1994).

Function of the GS/GOGAT cycle is controlled by modulation of the synthesis and catalytic activity of GS (Lee *et al.*, 1985). Under nitrogen-limiting conditions, synthesis of GS is elevated and the enzyme is in its unmodified, catalytically active form. When excess NH_4^+ is added to nitrogen-limited cultures, GS is rapidly adenylylated by GS adenylyltransferase (Schutt and Holzer, 1972; Wolheuter *et al.*, 1973; Kustu *et al.*, 1984) and thereby inactivated. In *glnE* mutant strains, which lack GS adenylyltransferase and are therefore unable to modify GS, the glutamate pool is rapidly depleted when nitrogen-limited cultures are subjected to a sudden NH_4^+ increase; the drop in glutamate is due to uncontrolled synthesis of glutamine, not to excretion into the medium (Kustu *et al.*, 1984).

GOGAT-deficient (*gltBD*) *E. coli* mutants have previously been constructed (Csonka *et al.*, 1994; Yan *et al.*, 1996). In the present study, we performed transposon insertion mutagenesis of a GOGAT-deficient (*gltBD*) strain to identify mutants that are complemented by the *Salmonella gogat* gene. In this study, we used *gltBD* mutant strains of *S. typhimurium* to complement the genes, since *S. typhimurium* GOGAT has not been reported yet. In this paper, the GOGAT gene has been cloned by complementation of a GOGAT-deficient *E. coli* mutant.

Materials and Methods

Bacterial strains and culture
Restriction enzymes and modification enzymes were purchased from BMS Korea and were used as recommended by the suppliers. Molecular marker kits for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Bio-Rad Laboratories Korea (Korea). Nylon membrane filters (Hybond-C) and ECL DNA hybridization kits were purchased from Amersham Korea (Korea). Other chemicals used were of the purest grade commercially

Table 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or characteristics[a]	Source
S. typhimurium	Wild type	MHWKG[b]
E. coli JRG72 mutant CH-gltB2767	(*gltB*, *sucA*1 *sup*E42 *iclR*::MudI) mutant (*gltB*2767)	This study
Plasmid		
pUC19	Cloning and expression vector, Amp[r]	Gibco BRL
pUC19C12	4.9-Kb DNA from *S. typhimurium* in pUC19, Amp[r]	This study
pUC19C13	4.9-Kb DNA from *S. typhimurium* in pUC19, Amp[r]	This study
pUC19C15	4.9-Kb DNA from *S. typhimurium* in pUC19, Amp[r]	This study

[a] Abbreviations for antibiotics : Amp, ampicillin.
[b] MHWKG, Ministry of Health and Welfare of Korean Government

available.

Strains derived from *S. typhimurium* LT2 (*S. typhimurium* ATCC 14028) were used as the source of the gene that codes for GOGAT (Table 1). Congenic strains *S. typhimurium* ATCC 14028 (wild-type) and GOGAT large subunit-deficient *E. coli* JRG72 (*glt*B, *suc*A1, *sup*E42, *iclR*) mutants CH-gltB2767 (*gltB*2767) and CH-gltB2768 (*gltB*2768) (Kim *et al.*, unpublished results) were used for complementation experiments; the latter two strains lack GOGAT activity. These strains are from our deposit in the Bacterial Collection Laboratory (BCL) of the Department of Biochemistry and Molecular Biology, Dongguk University, Kyungju, Kyungbuk, Korea (Kim C.H., 2003; Jin *et al.*, 2004). *S. typhimurium* growth experiments were performed aerobically at 37°C. The full-strength minimal medium was sodium-based N-C-medium, which contains (per liter) Na_2SO_4 (0.8 g), Na_2HPO_4 (11.0 g), $NaH_2PO_4 \cdot H_2O$ (4.8 g), $MgSO_4 \cdot 7H_2O$ (0.1 g), and NaCl (2.5 g). The diluted minimal medium was 0.2 × N-C-double-buffered (DB), which contains (per liter) Na_2SO_4 (0.16 g), Na_2HPO_4 (4.4 g), $NaH_2PO_4 \cdot H_2O$ (1.9 g), $MgSO_4 \cdot 7H_2O$ (0.1 g), and NaCl (0.5 g). Both media were supplemented with glycerol as a carbon source (0.2 or 0.4%, as indicated) and NH_4Cl (2 or 10 mM, as indicated) or proline (10 mM) as a nitrogen source and 1 mM KCl. Strains were grown overnight in nutrient broth medium and then subcultured into the medium used for a particular experiment, except that NH_4Cl was provided at 5 mM instead of 2 mM. After the cultures had reached saturation, cells were harvested by centrifugation, washed once with the medium to be used subsequently, and inoculated into warmed fresh medium to a low cell density ($O.D._{650}$ of 0.05 for cells grown on NH_4^+ and 0.15 for cells grown on proline).

E. coli JRG72 and *E. coli* JM109 were maintained in Luria broth (1% peptone, 0.5% yeast extract and 0.5% NaCl, pH 7.2), whereas JM109, JRG72/plasmid was maintained in Luria broth containing 40 μg/mL ampicillin. Cultures were preserved in 25% glycerol at -70°C. For selection of the cloned gene in *E. coli* JRG72, minimal glucose medium was also used.

DNA manipulation and cloning of gogat gene

Chromosomal DNA was extracted from *S. typhimurium* according to the method of Canosi *et al.* (Canosi *et al.*, 1978). Large-scale preparation of plasmid DNA was carried out as described (Ish-Horowicz and Burke, 1981). Restriction endonucleases (BRL, Gaithersberg, MD, USA) were used under the assay conditions recommended by the manufacturers. Agarose gel electrophoresis of DNA fragments was carried out in Tris-acetate buffer, pH 7.8, containing EDTA. DNA

from the gel was transferred onto Hybond Q membrane (Amersham) and used for hybridization. The DNA probe was nick-translated with [α-32P] dCTP and hybridized as described by Maniatis *et al.* (1982).

E. coli cell extracts were prepared from spheroplasts (Kaback *et al.*, 1995), which were lysed in 50 mM potassium phosphate buffer (pH 7.4). Cell membranes were removed by centrifugation at 35,000 × g, at 4°C, for 30 min. Chromosomal DNA from *S. typhimurium* was partially digested with *Sau*3AI. After removal of proteins, the resulting fragments were ligated to *Bam*HI-digested pUC19 DNA using T4 DNA ligase. *E. coli* JRG72 was transformed using these recombinant plasmids according to the method described by Mandel and Higa (1979). The transformants were selected for growth in minimal glucose medium.

S. typhimurium GOGAT expressed in a GOGAT large subunit-deficient *E. coli* mutant was screened by complementing the GOGAT large subunit-negative phenotype (Kim *et al.*, unpublished results). The *E. coli* GOGAT large subunit-deficient mutant, JRG72, was transformed with the plasmid library of the chromosomal inserts, selecting for the wild-type GOGAT phenotype (ability to grow on minimal glucose plates). Plasmids of pUC19C12, pUC19C13 and pUC19C15 were isolated.

DNA sequence analysis and Southern hybridization

DNA sequence was determined by the dideoxy chain reaction termination method with T7 DNA polymerase according to the manufacturer's instructions and as described (Ish-Horowicz and Burke, 1981). The nucleotide sequence of the *gogat* gene has been deposited in the GenBank database (Accession No. AF237961). Southern hybridization was also carried out as described (Maniatis *et al.*, 1982) in the supplier's instructions. DNA sequence information was analyzed through the National Center for Biotechnology Information, using the BLAST network service to search the Genbank database (Altschul *et al.*, 1990), and with MacVector sequence analysis software (version 6).

SDS-PAGE and analytical methods

SDS-PAGE was performed in 17% (w/v) gels with SDS by the method of Nevillei (1971). *E. coli* JRG72 strains carrying plasmids were cultured with or without 1 mM isopropyl β-D-thiogalactoside (IPTG) for induction. The sample buffer was 0.01 M Tris-HCl (pH 8.0) containing 2.5% SDS and in some cases 5% (vol/vol) β-mercaptoethanol. For relative molecular mass measurement, myosin (M_r 205,000), β-galactosidase (M_r 116,000), phosphorylase b (M_r

97,400), bovine serum albumin (M_r 66,000) and egg albumin (M_r 45,000) were used as standards. Protein content was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. Protein in the column eluates was routinely followed by the absorbance at 280 nm.

Results and Discussion

Cloning of the GOGAT large subunit gene from S. typhimurium in E. coli

S. typhimurium GOGAT expressed in a GOGAT-deficient *E. coli* mutant can complement the GOGAT-negative phenotype of the mutant (Oliver *et al.*, 1987; Saroja *et al.*, 1996). We have isolated the *gltB* gene, which encodes the *S. typhimurium* GOGAT large subunit, by complementation of *E. coli* strain JRG72 (*gltB, sucA1 supE42, iclR::MudI*) mutant CH-gltB2767 (*gltB2767*). The *E. coli* mutant strain CH-gltB2768 (*gltB2768::Mu* d) was also used to screen for the GOGAT large subunit gene (*gltB*) by complementation.

*Sau*3AI digests of genomic *S. typhimurium* DNA were ligated into the *Bam*HI site of plasmid pUC19, and the *E. coli* GOGAT-deficient mutant, JRG72, was

Table 2. Complementation activity of the cloned GOGAT large subunit gene of the plasmids pUC19C12, pUC19C13 and pUC19C15 in GOGAT-negative *E. coli* strain JRG72 (*gltB, sucA1 supE42 iclR::MudI*) mutant CH-gltB2767 (*gltB2767*)

plasmid	Complementation (+ or −)	Source
pUC19	−	Gibco BRL
pUC19C12	+	This study
pUC19C13	+	This study
pUC19C15	+	This study

transformed with the plasmid library of the chromosomal inserts, selecting for the wild-type GOGAT phenotype (ability to grow on NN minimal medium (Saroja *et al.*, 1996)). The libraries were screened for the expression of GOGAT by complementation of the phenotype of the *E. coli* GOGAT-deficient mutant. Approximately 3000 plasmids were screened in *E. coli* strain JRG72 (*gltB, sucA1 supE42 iclR::MudI*) mutant CH-gltB2767 (*gltB2767*). Three positive clones (named pUC19C12, pUC19C13 and pUC19C15) contained an identical 4.9 kb *Sau*3AI fragment as determined by restriction mapping and Southern hybridization; these transformants expressed GOGAT efficiently and constitutively (Table 2). Of the plasmids isolated from the three GOGAT clones, pUC19C12 was chosen for further study.

The plasmid pUC19C12 carrying the *gogat* gene on a 4.9-kb *Sau*3AI fragment was isolated (Fig. 1). The wild-type phenotype was restored in *E. coli* JRG72 (pUC19C12), which shows that functional *S. typhimurium* GOGAT can be expressed from the plasmid (Fig. 1). When Southern hybridization of cloned GOGAT gene was carried out with *S. typhimurium* chromosomal DNA, strong bands in only *S. typhimurium* and *E. coli* were detected, not in *V. parahaemolyticus*, *V. vulnificus*, *Enterobacter cloacae*, *P. aeruginosa* and *Proteus* sp. N 13838 (data not shown). This indicates that *S. typhimurium gogat* is structually similar to the gene in *E. coli*.

Nucleotide and amino acid sequences of S. typhimurium GOGAT large subunit

The cloned DNA in plasmid pUC19C12 carries *Bam*HI, 2 *Hinc*II, 4 *Hind*III, 2 *Kpn*I, 2 *Pst*I, 2 *Sal*I, 2 *Sma*I and 1 *Xba*I cleavage sites. The nucleotide
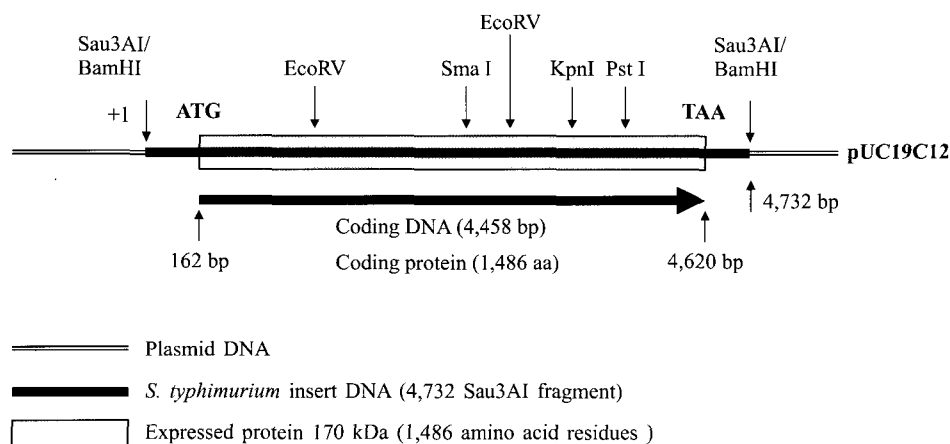

Fig. 1. Physical map of the *gogat* gene from *S. typhimurium*. The closed box regions correspond to cloned *S. typhimurium* DNA containing *gogat* gene. An arrow indicates the ORF direction of the *gogat* gene in plasmid pUC19C12. Closed and double lines indicate the plasmid-derived DNA and cloned DNA fragments, respectively.

A

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attgagattccctcttaaccgtatttaccaatgcgcaaaggacacaaagagggcgaatgcgaggtaagcgtatgatgcgccagccccgtc      90
                                      S.D.
gccatgctcttgctgtgcccgcgcgccgtcggttgggagagcgtcccgtagagcctggggaggttcactgatatgttgtacgataaatcc     180
                                                               M  L  Y  D  K  S
cttgagaaggataactgtggtttcggcctgatcgcccacatagaaggcgaacctagccacaaggtagtgcgtaccgccatacacgcgctg     270
 L  E  K  D  N  C  G  F  G  L  I  A  H  I  E  G  E  P  S  H  K  V  V  R  T  A  I  H  A  L
gcccgatgcagcaccgtggcgcgatccttgccgacggtaaaaccggcgacggttgcggcttgctgttacaaaaacctgaccgtttctt     360
 A  R  M  Q  H  R  G  A  I  L  A  D  G  K  T  G  D  G  C  G  L  L  L  Q  K  P  D  R  F  F
cgcatcgtggccgaagagcgcgggctggcgcttagccaaaaactacgccgtcgggatgatattttgaataaagatcctgaactggccgcc     450
 R  I  V  A  E  E  R  G  W  R  L  A  K  N  Y  A  V  G  M  I  F  L  N  K  D  P  E  L  A  A
gcgtcacgccatattgtcgaagaagaattacagcaggaaaccctgtcgattgtcggctggcgcgatgtgccaaccaatgaaggcgtgctc     540
 A  S  R  H  I  V  E  E  E  L  Q  Q  E  T  L  S  I  V  G  W  R  D  V  P  T  N  E  G  V  L
ggtgaaatcgccctctcctccctgccgcgtattgagcagattttgtcaacgctccggcgggctggcgtccgcgtgatatggagcgccgt     630
 G  E  I  A  L  S  S  L  P  R  I  E  Q  I  F  V  N  A  P  A  G  W  R  P  R  D  M  E  R  R
ctgtttatcgcccgccgacgcattgaaaaacgcctgcaggacgacaaagatttctacgtatgcagcctgtcgaacctggtcaacatttat     720
 L  F  I  A  R  R  R  I  E  K  R  L  Q  D  D  K  D  F  Y  V  C  S  L  S  N  L  V  N  I  Y
aaaggtctatgtatgccggcggatctgccgcgcttctacctggatcttgcggatctgcgtctggaatcggccatctgcctgttccaccag     810
 K  G  L  C  M  P  A  D  L  P  R  F  Y  L  D  L  A  D  L  R  L  E  S  A  I  C  L  F  H  Q
cgcttttccactaacaccgtgccgcgttggccgcttggccgcagccgttccgctacctggcgcataacggcgagattaacactatcaccggc     900
 R  F  S  T  N  T  V  P  R  W  P  L  A  Q  P  F  R  Y  L  A  H  N  G  E  I  N  T  I  T  G
aaccgccagtgggcgcgcgcccgtacctataaatccagacgccgctgatcccggatttacagtccgctgcgccgtttgttaacgaaacc     990
 N  R  Q  W  A  R  A  R  T  Y  K  F  Q  T  P  L  I  P  D  L  Q  S  A  A  P  F  V  N  E  T
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cccgcctggcagaacaaccccgatatggaccaggatctgcgcgccttctttgactttaactccatgcatatggagccgtgggacggtccg    1170
 P  A  W  Q  N  N  P  D  M  D  Q  D  L  R  A  F  F  D  F  N  S  M  H  M  E  P  W  D  G  P
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 A  G  I  V  M  S  D  G  R  F  A  A  C  N  L  D  R  N  G  L  R  P  A  R  Y  V  I  T  K  D
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 K  L  I  T  C  A  S  E  V  G  I  W  D  Y  Q  P  D  E  V  V  E  K  G  R  V  G  P  G  E  L
atggtgatcgacaccgcggcggacgttctgcattccgcgaaaccgatgacgacctgaaaagccgccatccatataaagagtggatg    1440
 M  V  I  D  T  R  G  G  R  I  L  H  S  A  E  T  D  D  D  L  K  S  R  H  P  Y  K  E  W  M
gagaaaaacgtccgccgcctggtgccgtttgaagagctgcctgatgaagaagtcggtagccgcgaactggacgatgacctgctcgccagc    1530
 E  K  N  V  R  R  L  V  P  F  E  E  L  P  D  E  E  V  G  S  R  E  L  D  D  D  L  L  A  S
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 P  I  D  P  L  R  E  A  H  V  M  S  L  A  T  S  I  G  R  E  M  N  V  F  C  E  A  E  G  Q
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cgtctggatatcacctcgacgtgaccgaaaccacactgaacgcgacggttaaggcgctgtgcgataaagcggaacagatggcgcgcaac    1980
 R  L  D  I  T  F  D  V  T  E  T  T  L  N  A  T  V  K  A  L  C  D  K  A  E  Q  M  V  R  N
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gaatacgccaaactggtcaatgagcgtccggcgtcaacgctgcgcgatctgctggcgatacatccagacggcgaggcggtgactatcgac    2700
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gaggttgagcccgccagtgagcgtgtttaaacgctttgataccgcagccatgtctatcggcgcattaagtccggaagcgcacgaagcgctg    2790
 E  V  E  P  A  S  E  L  F  K  R  F  D  T  A  A  M  S  I  G  A  L  S  P  E  A  H  E  A  L
gcggaagcgatgaacagcctcggccgcaactccaactccggcgaaggcggcgaagacccggccgcgctatggcaccaataaagtgtcgcgc    2880
 A  E  A  M  N  S  L  G  G  N  S  N  S  G  E  G  G  E  D  P  A  R  Y  G  T  N  K  V  S  R
atcaagcaggtggcttccggccgcttggcgtgacgccagcctacctggtcaatgccgatgtgattcagattaaagtcgcgcagggcgcg    2970
 I  K  Q  V  A  S  G  R  F  G  V  T  P  A  Y  L  V  N  A  D  V  I  Q  I  K  V  A  Q  G  A
aaaccgggcgaaggcggtcagttgccggggggataaagtcactccgtatatcgctaaactgcgctattcagtgccgggcgtgacgctgatc    3060
 K  P  G  E  G  G  Q  L  P  G  Q  K  V  T  P  Y  I  A  K  L  R  Y  S  V  P  G  V  T  L  I
tccccgccaccgcaccacgatatctactctatcgaggacttagcgcagctcattttcgatctcaagcaggtcaacccgaaggcgatgatc    3150
 S  P  P  P  H  H  D  I  Y  S  I  E  D  L  A  Q  L  I  F  D  L  K  Q  V  N  P  K  A  M  I
tccgtgaagctggtatccgaaccgggcgttcggcaccatcgcgacgccggtggcgaaagcctatgcggatctgatcactatcgccggctac    3240
 S  V  K  L  V  S  E  P  G  V  G  T  I  A  T  G  V  A  K  A  Y  A  D  L  I  T  I  A  G  Y
gacggcgggtaccggcgccagcccgctctcttcagtgaaatacgctggttgcccgtgggaactcggacttgttgaaacccagcaggcgctg    3330
 D  G  G  T  G  A  S  P  L  S  S  V  K  Y  A  G  C  P  W  E  L  G  L  V  E  T  Q  Q  A  L
gtcgccaacggtctgcgtcacaagattcgtctgcaggtggacggcggtctgaaaaccggcgtcgacatcattaaagcggcgattctcggt    3420
 V  A  N  G  L  R  H  K  I  R  L  Q  V  D  G  G  L  K  T  G  V  D  I  I  K  A  A  I  L  G
gcggaaagtttcggcttcggcaccggcccgatggtggcgctgggctgtaaataacctgcgtatctcgccacctgaataactgcgcaaccggc    3510
 A  E  S  F  G  F  G  T  G  P  M  V  A  L  G  C  K  Y  L  R  I  C  H  L  N  N  C  A  T  G
gtagcaactcaggatgacaagctgcgtaagaaccactatcacggcctgccgttcaaagtgactaactactttgaatttatcgcccgcgaa    3600
 V  A  T  Q  D  D  K  L  R  K  N  H  Y  H  G  L  P  F  K  V  T  N  Y  F  E  F  I  A  R  E
gtgcgcgagctgatggcttcgcttggccgtgacccgccctggtggatttgattggccgcaccgacctgctgaaaagagctggagggcttcacc    3690
 V  R  E  L  M  A  S  L  G  V  T  R  L  V  D  L  I  G  R  T  D  L  L  K  E  L  E  G  F  T
gctaaacagcagaagctggcgctgtctcgtctactggaaaccgtgaacctcatccgggtaaagcgctgtactgcaccgagaataacccg    3780
 A  K  Q  Q  K  L  A  L  S  R  L  L  E  T  A  E  P  H  P  G  K  A  L  Y  C  T  E  N  N  P
ccgtttgataacggcgtgctgaatgcgcaactgttgcagcaggcgaaacctttgtcgatgcgcgcccagagcaaaaccttctggttcgac    3870
 P  F  D  N  G  V  L  N  A  Q  L  L  Q  Q  A  K  P  F  V  D  A  R  Q  S  K  T  F  W  F  D
atccgcaacacccgatcgttccgtcggcgcgtcgctttccggctacatcgcacagaccccacggcgatcaggggctggcttccgatccgatt    3960
 I  R  N  T  D  R  S  V  G  A  S  L  S  G  Y  I  A  Q  T  H  G  D  Q  G  L  A  S  D  P  I
aaagcacacttcagcggtaccgcaggccagagctttggcgtgtggaacgcgggccggcgtagagttatatctgactggcgatgccaacgac    4050
 K  A  H  F  S  G  T  A  G  Q  S  F  G  V  W  N  A  G  G  V  E  L  Y  L  T  G  D  A  N  D
tatgtcggtaaaggtatggcaggccggtttgattgccatcagaccaccgtaggttccgcctcctcagtcataaagccagcattatcggc    4140
 Y  V  G  K  G  M  A  G  G  L  I  A  I  R  P  P  V  G  S  A  F  L  S  H  K  A  S  I  I  G
aacacctgcctgtatggagccaccggtggccgtctgtacgcggcgggccgcgcgggcgaacgtttcggcgtgcgtaactccgggcgcgatt    4230
 N  T  C  L  Y  G  A  T  G  G  R  L  Y  A  A  G  R  A  G  E  R  F  G  V  R  N  S  G  A  I
accgtagtagagggcatcggcgacaacggctgtgaatacatgaccggcggccatcgtctgcgtgttgggtaaaaccggcgttaacttcggc    4320
 T  V  V  E  G  I  G  D  N  G  C  E  Y  M  T  G  G  I  V  C  V  L  G  K  T  G  V  N  F  G
gcggggatgaccggcggcttcgcctatgtgctggacgaagatggcgaattccgtaaacgcgtgaacccggaacctggtagaagtactggac    4410
 A  G  M  T  G  G  F  A  Y  V  L  D  E  D  G  E  F  R  K  R  V  N  P  E  L  V  E  V  L  D
gttgattcgctggctatccacgaagagcatctgcgcggtctgatcaccgagcacgtgcagcatactggttcgcaacgcggcgaagagatc    4500
 V  D  S  R  W  S  S  F  S  T  Q  F  A  L  V  K  P  K  S  S  D  V  K  A  L  L  G  H  R  S  R
ctgtcaggtggtcaagcttctcaacccaattcgcgttagttaagcccaagtccagcgatgtcaaagcccgtgtgggcaccgtagtcgt    4590
 L  S  R  W  S  S  F  S  T  Q  F  A  L  V  K  P  K  S  S  D  V  K  A  L  L  G  H  R  S  R
agtgcggcagaattgccgcgtgcaggcgcagtaaggaattgaaatgagccagaatgtttatcaatttatcgacctgcagcgcgttgatccg    4680
 S  A  A  E  L  R  V  Q  A  Q  END
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```
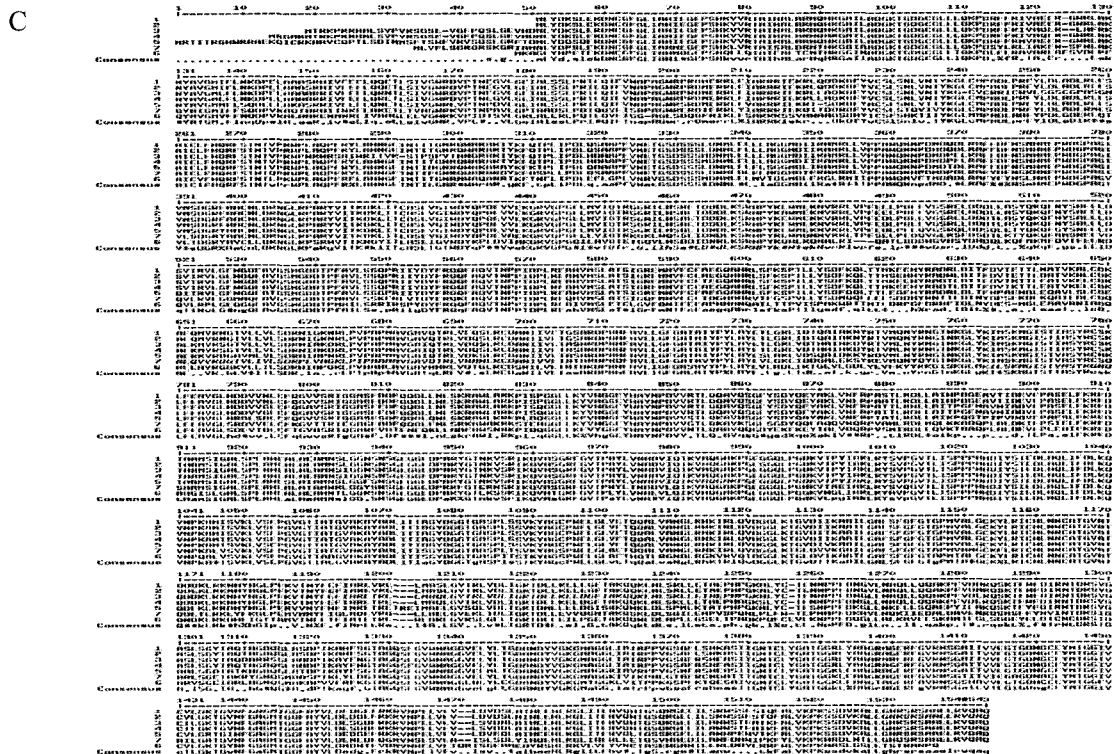
B

Fig. 2. Nucleotide sequence and comparison of deduced amino acid sequence of *S. typhimurium gogat* gene. (A) The 4,733 bp sequence contains the 5'-starting region of the *gogat* gene. The ribosome binding sequence is indicated as SD. The nucleotide at position 1 corresponds to the first nucleotide in the *Hind*III recognition sequence. (B) Comparison of DNA sequence corresponding to the catalytic domain of *S. typhimurium ogdh* with that of *E. coli*. Consensus nucleotides and mismatched nucleotides are indicated as red and blue letters, respectively. (C) Comparison of the deduced amino acid sequence corresponding to the catalytic domain of *S. typhimurium* GOGAT with those of the bacterial GOGATs that were reported. Consensus nucleotides and mismatched nucleotides are indicated as red and blue letters, respectively. 1, *S. typhimurium* (this research); 2, *S. enterica* serovar Typhi (GenBank No: AE016845); 3, *E. coli* (GenBank No: M18748); 4, *Shigella flexneri* (GenBank No: AE016989); 5, *Yersinia pestis* (GenBank No: AJ414157); 6, *Vibrio vulnificus* (GenBank No: BA000037); 7, *Pseudomonas aeruginosa* (GenBank No: AE004916).

sequence of the *gogat* gene was determined using subcloned DNA fragments from pUC19C12. The *gogat* gene sequence contains an open reading frame (ORF) consisting 4,458 bp (including ATG) encoding a protein of 1,486 amino acids (Fig. 2A). This open reading frame is preceded by a putative ribosome-binding site, d(GGAAGG), 14 bp from the ATG translational start codon. The nucleotide sequence of the ORF of *S. typhimurium* GOGAT shows 86% identity to *E. coli gogat*, which encodes the large subunit (Saroja *et al.*, 1996) (Fig. 2B).

The amino acid sequence of GOGAT, as deduced from the nucleotide sequence, is presently known from *E. coli* (Saroja *et al.*, 1996), *S. enterica* serovar Typhi (Deng *et al.*, 2003), *Shigella flexneri* (Wei *et al.*, 2003), *Yersinia pestis* (Parkhill *et al.*, 2001), *Vibrio vulnificus* (Chen *et al.*, 2003) and *Pseudomonas aeruginosa* (Stover *et al.*, 2000). When these sequences were compared with the translated *S. typhimurium* GOGAT region, they showed significant sequence

homology; the identity between *S. typhimurium* and *E. coli* GOGAT is 91% (Fig. 2C). When the *S. typhimurium* GOGAT was compared with the other bacterial GOGAT large subunits (*glt*B gene) listed above, approximately 81-97% similarities were found (Fig. 2C). Because the *S. typhimurium* GOGAT region can complement an *E. coli* mutant defective in GOGAT, the GOGAT large subunit components of these two bacteria must also be functionally similar.

It was previously known that the full sequence of *E. coli* GOGAT is composed of large and small subunits, in total 6.3 kb in length (Oliver *et al.*, 1987). Of these, the large *E. coli* subunit is 4860 bp, and nucleotides 236-4845 comprise the large subunit's ORF. The sequence analysis showed that this region corresponds to the ORF of *S. typhimurium* GOGAT (+162 to +4,620 nt).

It was known that bacterial (NADPH-GOGAT) and plant [(ferredoxin (Fd) and NAD(P)H-GOGAT] types of GOGAT all contain flavins (FMN and FAD) and

FMN-binding domain

```
1  967   ISPP PHHDIYSIEDLAQLI FDLKQVNPKAMISVK LVSEPGVGTIATGVA KAYADLITIAGYDGG  1029
2  967   ISPP PHHDIYSIEDLAQLI FDLKQVNPKAMISVK LVSEPGVGTIATGVA KAYADLITIAGYDGG  1029
3  995   ISPP PHHDIYSIEDLAQLI FDLKQVNPKAMISVK LVSEPGVGTIATGVA KAYADLITIAGYDGG  1057
4  1003  ISPP PHHDIYSIEDLAQLI FDLKQVNPKAMISVK LVSEPGVGTIATGVA KAYADLITIAGYDGG  1065
5  1016  ISPP PHHDIYSIEDLAQLI FDLKQVNPKAMISVK LVSEPGVGTIATGVA KAYADLITIAGYDGG  1078
6  984   ISPP PHHDIYSIEDLAQLI FDLKQVNPKALVSVK LVSEPGVGTIATGVA KAYADLITISGYDGG  1046
7  966   ISPP PHHDIYSIEDLAQLI FDLKQVNPQALVSVK LVSEPGVGTIAAGVA KAYADLITISGYDGG  1028
8  1029  ISPP PHHDIYSIEDLAELI HDLKNANREARINVK LVSEVGVGTIAAGVA KAHADVVLVSGYDGG  1093
9  1017  ISPP PHHDIYSIEDLAQLI YDLKQINPDAKVTVK LVSRSGIGTIAAGVA KANAKIILISGNSGG  1081
        **** ************* **  *** *  *   ** ***  * **** *** ** *    *  **
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3Fe-4S cluster

```
1  1030  TGASPLSSVKYAGCP WELGLVETQQALVAN GLRHKIRLQVDGGLK TGVDIIKAAILGAES FGFGTGPMVALGCKY LRICHLNNCATGVAT  1119
2  1030  TGASPLSSVKYAGCP WELGLVETQQALVAN GLRHKIRLQVDGGLK TGVDIIKAAILGAES FGFGTGPMVALGCKY LRICHLNNCATGVAT  1119
3  1058  TGASPLSSVKYAGCP WELGLVETQQALVAN GLRHKIRLQVDGGLK TGVDIIKAAILGAES FGFGTGPMVALGCKY LRICHLNNCATGVAT  1147
4  1066  TGASPLSSVKYAGCP WELGLVETQQALVAN GLRHKIRLQVDGGLK TGVDIIKAAILGAES FGFGTGPMVALGCKY LRICHLNNCATGVAT  1155
5  1079  TGASPLSSVKYAGCP WELGLVETQQALVAN GLRHKIRLQVDGGLK TGVDIVKAAILGAES FGFGTGPMVALGCKY LRICHLNNCATGVAT  1168
6  1047  TAASPLTSVKYAGSP WELGLAETQQALVAN GLRHKIRLQVDGGLK TGLDVVKGAILGAES FGFGTAPMVAMGCKF LRICHLNNCATGVAT  1136
7  1029  TGASPITSIKYAGSP WELGLAETHQTLRGN DLRGKVRVQTDGGLK TGLDVIKAAILGAES FGFGTAPMIALGCKY LRICHLNNCATGVAT  1118
8  1094  TGASPQTSIKHAGLP WELGLAETHQTLVLN NLRSRIVVETDGQMK TGRDVAIAALLGAEE FGFSTAPLVSLGCIM MRACHLNTCPVGIAT  1168
9  1082  TGASPQTSIKFAGLP WEMGLSEVHQVLTLN RLRHRVRLRTDGGLK TGRDIVIAAMLGAEE FGIGTASLIAMGCIM VRQCHSNTCPVGVCV  1156
        *****  *  ** *  **  ** *  *  *  **   ** *** *   ** ****  **  *      **   * ** *  *  *
```

Fig. 3. Comparison of the flavin-binding sites and 3Fe-4S centers in the C-terminal regions of various GOGATs. The FMN-binding domain and 3Fe-4S center are underlined. The aspartic acid and lysine/arginine residues involved in the binding of the rivityl chain of FMN are marked in bold print. The 3 cysteines of the iron-sulfur cluster are denoted by bold print. Asterisks indicate identical amino acid residues in all the sequences. GOGATs of *E. coli* (Oliver *et al.*, 1987), *S. enterica* serovar Typhi (Deng and Liou, 2003), *S. flexneri* (Wei *et al.*, 2003), *Y. pestis* (Parkhill *et al.*, 2001), *V. vulnificus* (Chen *et al.*, 2003), *P. aeruginosa* (Stover *et al.*, 2000), *Syncechocystis* sp. PCC 6803 (Navarro *et al.*, 1995) and *Azospirillum brasilense* (Pelanda *et al.*, 1993) were compared.
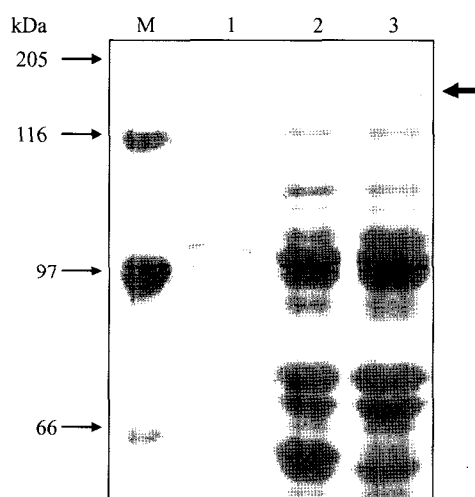

Fig. 4. SDS-PAGE of the *E. coli* extracts carrying the functional GOGAT having complementation activity. Total cell extracts were fractionated on an SDS-10% (wt/vol) acrylamide gel and stained for protein with Coomassie blue R250. Lanes: 1, *E. coli* strain JRG72 (*glt*B, *suc*A1 *sup*E42 *icl*R::MudI) mutant CH-gltB2767 (*gltB2767*); 2, *E. coli* strain JRG72 (pUC19C12) without IPTG; 3, *E. coli* JRG72 (pUC19C12) with IPTG. Approximately 20 µg of protein were loaded in each lane except for lane 1 (10 µg protein). At left are indicated the molecular weight positions in kD. The arrow indicates a ~170 kDa glutamate synthase.

iron-sulfur centers (4Fe-4S or 3Fe-4S) (Navarro *et al.*, 1995). Flavin-binding sites and 3Fe-4S centers are found in the C-terminal regions of GOGAT from *E. coli* (Saroja *et al.*, 1996), *S. enterica* serovar Typhi (Deng *et al.*, 2003), *S. flexneri* (Wei *et al.*, 2003), *Y. pestis* (Parkhill *et al.*, 2001), *V. vulnificus* (Chen *et*

al., 2003), *P. aeruginosa* (Stover *et al.*, 2000), *Syncechocystis* sp. PCC 6803 (Navarro *et al.*, 1995) and *Azospirillum brasilense* (Pelanda *et al.*, 1993). The 3Fe-4S cluster is likely located in a cysteine-rich region in *S. typhimurium* GOGAT (Cys-1102, Cys-1108 and Cys-1113). Similar cysteine clusters (CX_5CX_4C) are also observed in fumarate reductase and succinate dehydroganase which carry 3Fe-4S clusters (Johnson *et al.*, 1989; Manodor *et al.*, 1992). *S. typhimurium* GOGAT also contains 13 glycines in the 999-1086 region (Fig. 3). In fact, a glycine-rich region is required for formation of the β-α-β secondary structure in flavoproteins (Navarro *et al.*, 1995).

Functional expression of S. typhimurium GOGAT in E. coli

Expression of functional *gogat* (*glt*B) gene product from recombinant plasmids was similarly determined. Expression of the *S. typhimurium* protein results in complementation activity, and a ~170-kDa polypeptide was the most abundant protein in cytoplasmic extracts of *E. coli* JRG72 (pUC19C12). This polypeptide was not found in *E. coli* strain JRG72 (*glt*B, *suc*A1 *sup*E42 *icl*R::MudI) mutant CH-gltB2767 (*gltB2767*) (Fig. 4). It is known that *E. coli* GOGAT has two non-identical subunits with molecular masses of 166 kDa and 52 kDa (Saroja *et al.*, 1996). GOGATs of plant, algae and cyanobacteria, however, produce Fd-dependent glutamate synthase as a 130-170 kDa monomeric protein (Navarro *et al.*, 1995). *Saccharomyces cerevisiae* GOGAT is composed of a heterodimer of 166 kDa and 56 kDa subunits (Valenzuela *et al.*, 1998). NADPH-dependent glutamate synthase of

bacteria is a dimer of iron-sulfur flavoproteins and is composed of a large subunit of 135-175 kDa and a small subunit of 51-55 kDa (Suzuki and Rothstein, 1997). Therefore, it was concluded that the *S. typhimurium* GOGAT is quite similar to the GOGAT enzymes reported to date. Work in progress is characterizing the molecular structure to explain the complementation activity.

Acknowledgment

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