Plant Molecular Biology **27**: 753–767, 1995.

Existence of two ferredoxin-glutamate synthases in the cyanobacterium *Synechocystis* sp. PCC 6803. Isolation and insertional inactivation of *gltB* and *gltS* genes

Francisco Navarro, Sebastián Chávez, Pedro Candau and Francisco J. Florencio*
*Instituto de Bioquímica Vegetal y Fotosíntesis and Departamento de Bioquímica Vegetal y Biología Molecular, Universidad de Sevilla-CSIC, Apdo. 1113; 41080-Sevilla, Spain (*author for correspondence)*

Received 4 August 1994; accepted in revised form 23 December 1994

Key words: cyanobacteria, ferredoxin, *gltB*, *gltS*, glutamate synthase, *Synechocystis* sp. PCC 6803

Abstract

The first two genes of ferredoxin-dependent glutamate synthase (Fd-GOGAT) from a prokaryotic organism, the cyanobacterium *Synechocystis* sp. PCC 6803, were cloned in *Escherichia coli*. Partial sequencing of the cloned genomic DNA, of the 6.3 kb *Hin*d III and 9.3 kb *Cla* I fragments, confirmed the existence of two different genes coding for glutamate synthases, named *gltB* and *gltS*. The *gltB* gene was completely sequenced and encodes for a polypeptide of 1550 amino acid residues (M_r 168 964). Comparative analysis of the *gltB* deduced amino acid sequence against other glutamate synthases shows a higher identity with the alfalfa NADH-GOGAT (55.2%) than with the corresponding Fd-GOGAT from the higher plants maize and spinach (about 43%), the red alga *Antithamnion* sp. (42%) or with the NADPH-GOGAT of bacterial source, such as *Escherichia coli* (41%) and *Azospirillum brasilense* (45%). The detailed analysis of *Synechocystis gltB* deduced amino acid sequence shows strongly conserved regions that have been assigned to the 3Fe-4S cluster (CX_5CHX_3C), the FMN-binding domain and the glutamine-amide transferase domain. Insertional inactivation of *gltB* and *gltS* genes revealed that both genes code for ferredoxin-dependent glutamate synthases which were nonessential for *Synechocystis* growth, as shown by the ferredoxin-dependent glutamate synthase activity and western-blot analysis of the mutant strains.

Introduction

Ammonium assimilation in photosynthetic organisms occurs predominantly through the glutamine synthetase (GS)-glutamate synthase (GOGAT) pathway, commonly known as GS-GOGAT cycle, which produces a net molecule of glutamate [36].

Two types of glutamate synthases have been described in higher plants and algae; one uses pyridine nucleotides [NAD(P)H-GOGAT], and the other ferredoxin (Fd-GOGAT), as electron

The nucleotide sequence data reported will appear in the EMBL and GenBank Nucleotide Sequence Databases under the accession number X80485.

donors [49]. Both types of glutamate synthases seem to be confined to plastids, being the Fd-GOGAT the pre-eminent form in the chloroplast [6, 29, 48], while NADH-GOGAT has been localized in non-photosynthetic tissues, such as roots [50] and nodules [1, 13, 19]. By contrast, in bacteria only NAD(P)H-GOGAT has been identified [21, 37, 51, 54], with the exception of cyanobacteria which only contain ferredoxin-dependent glutamate synthase [31].

Ferredoxin-GOGAT has been purified to electrophoretic homogeneity and characterized from different photosynthetic sources, such as spinach [22], maize [45], rice [50] and tomato [3], the green alga *Chlamydomonas reinhardtii* [18] and the cyanobacterium *Synechococcus* sp. PCC 6301 [31]. Fd-GOGAT is a monomeric protein ranging from 145 to 170 kDa in size, containing FAD and FMN and a distinctive iron-sulfur cluster [3Fe-4S] [18, 22, 23, 27]. However, in *Synechococcus* Fd-GOGAT, no FAD was found in the purified protein [31].

A full cDNA encoding a Fd-GOGAT (*gltS* gene) has only been isolated from maize [45] and in partial form from spinach [38], tobacco [56] and barley [4]. Recently, a gene, (*glsF*), encoding also a Fd-GOGAT, was found in the chloroplast DNA of the red alga *Antithamnion* sp., differing from the nuclear location of the plant Fd-GOGAT gene [52]. In addition, the gene encoding the NADH-GOGAT of alfalfa has been cloned and sequenced, showing clear differences in size and amino acid sequence with respect to the ferredoxin-dependent glutamate synthase genes [19]. The corresponding protein (NAD-(P)H-GOGAT) has been purified from other photosynthetic sources [1, 5, 14, 20, 24, 32].

In bacteria, NADPH-GOGAT is composed of two different subunits, encoded by *gltD* (small subunit) and *gltB* (large subunit) genes, which have been extensively characterized in *E. coli* and *A. brasilense* [40, 42], but no information exists about Fd-GOGAT genes in prokaryotes that could be used to elucidate the origins of the plant Fd-GOGAT and NADH-GOGAT [19, 52].

To address not only the role of glutamate synthase in ammonium assimilation in cyanobacteria, but also if the Fd-GOGAT gene could be related with the gene coding for Fd-GOGAT in higher plants, we choose the unicellular cyanobacterium *Synechocystis* sp. PCC 6803, whose genes encoding glutamine synthetase and glutamate dehydrogenase have been previously cloned, as a good system to obtain mutants affected in the ammonium assimilation pathway [12, 35].

In this study, we demonstrate for the first time the existence of two different genes coding for Fd-GOGAT (*gltB* and *gltS*) in a cyanobacterium. These genes are actively expressed in normal growth conditions and can be independently inactivated without affecting the *Synechocystis* 6803 cell growth. In addition, the deduced amino acid sequence of *gltB* supports the view that *gltB* gene is more related to the gene coding for NADH-glutamate synthase than to its Fd-GOGAT counterparts.

Materials and methods

Bacterial strains, plasmids and growth conditions

E. coli strain DH5α (Bethesda Research Laboratories), used for all plasmid constructions; HB101 [7] and MC1061 [46], used for gene library constructions, were grown in Luria broth [46] supplemented with ampicillin at a final concentration of 100 μg/ml.

All the plasmids used were pBluescript II SK(+) derivatives. pFN3 and pFN5 contained 3.9 kb and 6.3 kb *Synechocystis* DNA fragments that include part or the whole *gltB* gene; pFN4 and pFN7 have 4.9 kb and 9.3 kb DNA inserts containing part or the complete *gltS* gene.

Synechocystis sp. PCC 6803 and its mutant strains were cultured at 30 °C with shaking in BG11 medium containing nitrate as nitrogen source [44] and bubbled with a continuous stream of 1.5% (v/v) CO_2 in air when used to obtain cell-free extract preparations. For plate cultures, BG11 liquid medium was supplemented with 1% (w/v) agar. Kanamycin and chloramphenicol were added to a final concentration of 50 μg/ml and 20 μg/ml, respectively, when required.

DNA manipulations

Total *Synechocystis* DNA was isolated as described [9], supplementing the culture with penicillin G at 100 μg/ml 20 h before the extraction. All DNA manipulations and *E. coli* transformations were performed following standard procedures [46]. DNA fragments were purified from the agarose gels using the GeneClean Kit (Bio 101 Inc.). For Southern hybridization, DNA was digested, and fragments were electrophoresed in 0.7% agarose gels using the Tris-borate-EDTA buffer system [46]. DNA was transferred to Z-probe membranes (Bio-Rad) using vacuum and Southern blot hybridization was performed as described [2]. Colony hybridization was carried out as indicated [46]. In all cases hybridizations were performed in 5 × SSC (1 × SSC is 0.15 M NaCl in 1.5 mM sodium citrate) at 68 °C, except when the probe was the 1.2 kb fragment generated by polymerase chain reaction, at 65 °C. DNA probes were ^{32}P-labelled with a nick translation kit (Boehringer Mannhein) or a random-primer kit (Pharmacia) using [α-^{32}P]-dCTP (3000 Ci/mmol).

PCR techniques

PCR (Polymerase Chain Reaction) amplifications were carried out as described in [46], using a Gene ATAQ Controller from Pharmacia. The two oligonucleotides selected from the comparison of the deduced amino acid sequences of maize *gltS* [45] and *E. coli gltB* [40] genes were:

5′-CCT(CGA)CAT(C)CAT(C)GAT(CA)A-TT(C)TA-3′ and 3′-ACA(G)CTT(C)ATA(G)-TACTGA(TCG)CC-5′.

The oligonucleotide concentrations were calculated by measuring at 260 nm as described [46].

Insertional mutagenesis of gltB *and* gltS *genes of* Synechocystis

To mutate the *gltB* gene, a 894 bp *Hinc* II internal *gltB* fragment was replaced by a 1.9 kb fragment containing a chloramphenicol resistance gene from pKT210 [16]. The new plasmids were named pFN10+ and pFN10−, depending on orientation of the resistance cassettes respect to the *gltB* gene. For *gltS* disruption, a 1.3 kb fragment containing a kanamycin resistance gene from Tn5 [16] was cloned into an *Xba* I site of *gltS* in both orientations, generating pFN11+ and pFN11−. All these plasmids were used to transform *Synechocystis* WT strain by the method described [11].

DNA sequence determination and analysis

Nested deletions of relevant plasmids were performed by using a double-stranded Nested Deletion Kit from Pharmacia. Complete sequence of both strands was determined by the dideoxy chain termination method [47], using Sequenase 2.0 (USB). Computer sequence analysis was carried out using the Genetics Computer Group (GCG) software package [15]. Sequence analysis comparison was made by using the FASTA program [41] and alignments were produced with the PileUp program that uses the algorithm of Needleman and Wunsch [39] with a gap penalty of 3 and a length penalty weight of 0.1, and optimized by visual analysis. Percentage of identity was calculated from the conserved regions inferred of the Pileup alignment.

Ferredoxin-dependent glutamate synthase assays

Fd-glutamate synthase activity was determined by measuring glutamate formation as previously described [31] in cell-free extracts of *Synechocystis* strains prepared in 20 mM potassium phosphate buffer, pH 7.5, containing 10 mM 2-mercaptoethanol and 1 mM EDTA (buffer A), supplemented with phenylmethylsulfonylfluoride (PMSF) at a final concentration of 1 mM. Purified *Synechocystis* ferredoxin was used as electron donor. The reaction was started by adding 0.8 mg of sodium dithionite freshly dissolved in 0.1 ml of 0.12 M $NaHCO_3$ and was stopped, after 20 min

at 30 °C, by mixing 0.25 ml of the reaction mixture with 0.15 ml of 1 M HCl.

One unit of enzyme activity corresponds to the formation of 1 μmol of glutamate per minute.

Protein concentrations were determined by the method of Bradford [8] using ovalbumin as standard.

Purification of ferredoxin-dependent glutamate synthases

For enzyme purification, 1.5 g of frozen *Synechocystis* cells either WT (wild type) or mutant strains, SFN1+ and SFN2+, were thawed in 5 ml of buffer A containing 1 mM PMSF. The mixtures were disrupted by sonication (20 kHz, 75 W) at 0 °C for 30 s (in 15 s periods) in a Branson sonifyer model 250.

The homogenate was cleared by centrifugation at 23,000 × ***g*** for 15 min and the resulting supernatant was loaded onto a DEAE-cellulose column (1 cm × 10 cm) and washed with buffer A. Elution of glutamate synthases was carried out by applying a linear gradient (0.1–0.5 M NaCl) in 40 ml of the same buffer. GltB and GltS eluted as single peaks at about 0.2–0.24 M NaCl and 0.22–0.28 M of NaCl respectively. The active fractions were pooled, concentrated using a microconcentrator microsep (Filtron 30 K molecular weight cutoff) and analysed by SDS-PAGE.

Polyacrylamide gel electrophoresis and western-blot procedures

Partial enzyme purification was followed by SDS-PAGE according to the method described in [28], using 6.5% acrylamide slabs gels. Protein bands were stained with 0.25% Coomassie brilliant blue R-250 (Sigma Chemical Co. St. Louis, MO). For western-blot analysis, proteins, after SDS-PAGE, were electrotransferred to a nitrocellulose sheet, incubated with antiserum against *Synechococcus* PCC 6301 Fd-GOGAT (diluted 1:100 in TBS buffer, containing 5% dry powdered milk and 0.05% (v/v) Tween 20), washed three times for 5 min and then incubated for 1 h with 1:1000 diluted horseradish-peroxidase-conjugated sheep anti-rabbit antibody. Membranes were subsequently washed and the reaction was visualized by immersion in the developing standard solution.

Results and discussion

Cloning of gltB *and* gltS *genes*

A PCR amplification step was followed to obtain an internal fragment of the gene encoding the Fd-GOGAT from *Synechocystis*. For that purpose we designed a direct primer corresponding to the amino acid residues 1097–1102 of maize Fd-GOGAT [45] and to residues 998–1003 of the large subunit of the NADPH-GOGAT from *E. coli* [40] respectively, and as reverse primer the corresponding to residues 1494–1499 and 1395–1400 from the same glutamate synthases (see Materials and methods). The PCR amplification products, using *Synechocystis* genomic DNA, were separated by electrophoresis in 1% agarose gel, yielding two bands of 1.2 kb and 0.7 kb. The 1.2 kb fragment had a size according with the fragments of maize and *E. coli* glutamate synthase genes between the two oligonucleotides used. The purified 1.2 kb DNA band was digested with *Hinc* II, giving three fragments of 1.2 kb, 0.7 kb and 0.5 kb, four fragments with *Bst* XI (1, 0.7, 0.5, and 0.2 kb) and more than five when the fragment was digested with *Sau* 3AI, which gave a total size higher than 1.2 kb. These results suggested that two different fragments of 1.2 kb were amplified, probably corresponding to different genes coding for glutamate synthases, although there was no previous evidence for the existence of two glutamate synthases in cyanobacteria [31]. The 1.2 kb PCR product was used as a probe in a Southern blot hybridization with total *Synechocystis* DNA, shown in Fig. 1. Since the probe was of 1.2 kb and total DNA digested with *Dra* I gave four hybridization bands, with three of them above 1.2 kb, and with *Hae* II, also appeared

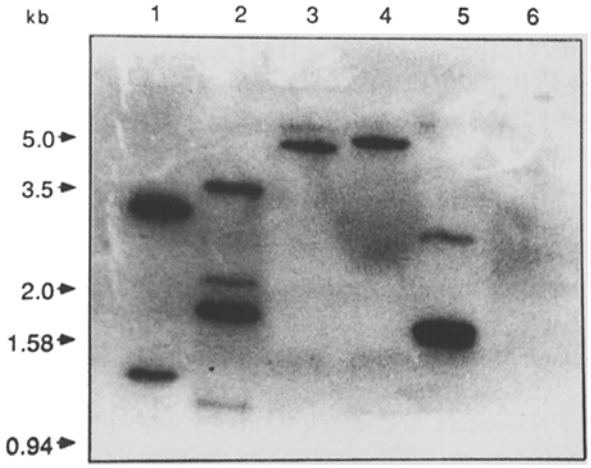


Fig. 1. Southern blot analysis of *Synechocystis* genomic DNA. *Synechocystis* genomic DNA (3 μg) was digested with *Hinc* II (lane 1), *Dra* I (lane 2), *Sma* I (lane 3), *Hin*d III (lane 4), *Hae* II (lane 5), and *Xmn* I (lane 6), separated by 0.7% agarose gel electrophoresis and then transferred to nylon membranes. The filter was hybridized using the 1.2 kb PCR product as a probe.

three bands higher than the probe (Fig. 1, lanes 2 and 5), we concluded that the probe hybridized with more than one gene.

Based on these results and taking into account that the PCR amplification generated two different fragments of 1.2 kb, we inferred that only two genes hybridized with the probes. Thus, we proceeded to clone those genes and for that purpose we isolated the 4.9 kb *Sma* I and the 3.9 kb *Xmn* I fragments by constructing chromosomal libraries of *Sma* I and *Xmn* I fragments of these sizes in the plasmid pBluescript II SK(+) and screening by colony hybridization in *E. coli* MC1061, using the 1.2 kb PCR fragment as a probe. Two clones containing the *Xmn* I fragment (plasmid pFN2 and pFN3) and one containing the *Sma* I fragment (pFN4) were selected, on the basis of their differential restriction maps. Partial nucleotide sequences of pFN3 and pFN4 revealed an open reading frame in each one, whose deduced amino acids sequences showed strong similarities with the internal amino acid region of the maize and *E. coli* glutamate synthases located between the two oligonucleotides used for PCR amplification (data not shown). This confirmed the existence of two different genes for glutamate synthases in *Synechocystis* sp. PCC 6803, which we named *gltB* and *gltS*. Since the molecular mass of Fd-GOGAT from *Synechococcus* 6301 [31] and maize [45] is ca. 160 kDa, we expected that both genes should be at least 5.5 kb in length. Using as a probe internal regions of both of them, a new Southern blot with *Synechocystis* genomic DNA was carried out to clone the whole *gltB* and *gltS* genes. The Southern blot resulted in a cross-

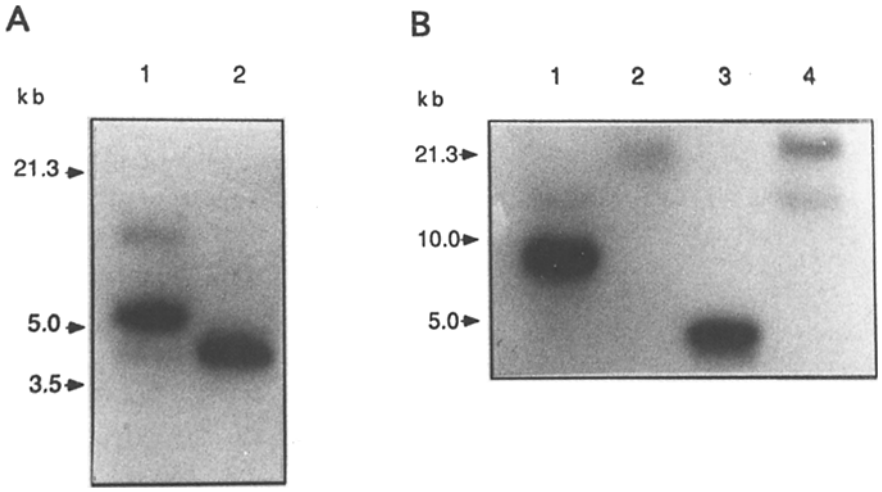


Fig. 2. Identification of the *gltB* and *gltS* genes in *Synechocystis* DNA. Hybridization of total *Synechocystis* DNA. A. *Hin*d III (lane 1), *Bst* XI (lane 2). B. *Cla* I (lane 1), *Bam* HI (lane 2), *Bst* XI (lane 3), and *Bgl* II (lane 4). The probes used for hybridization in panels A and B, are indicated in Fig. 3, probes I and II, respectively.

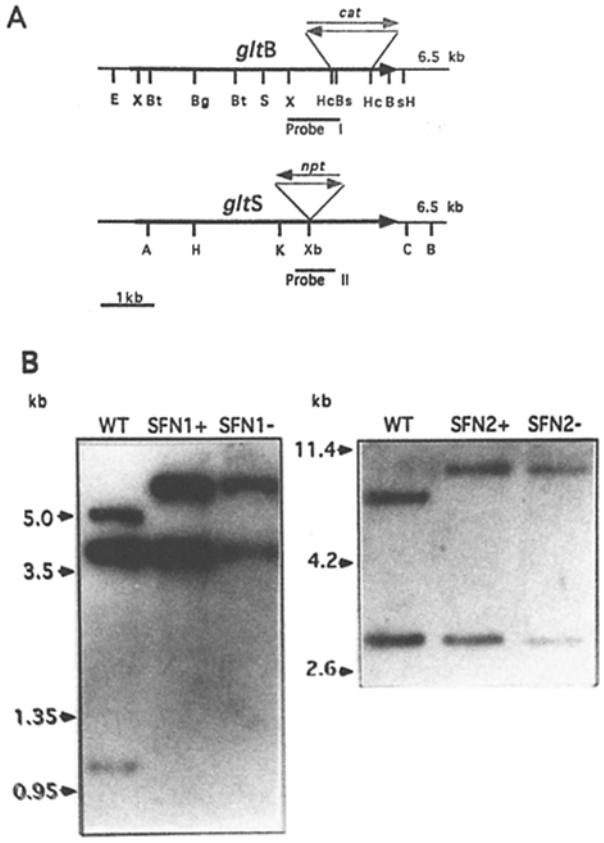


Fig. 3. Disruption construct and Southern blot analysis of SFN1 and SFN2 genomic disruption. A. Restriction map of the *gltB* and *gltS* regions in wild-type *Synechocystis* sp. PCC 6803. Insertion of a *cat* and *npt* gene cassettes at the locations indicated generated mutant strains SFN1 and SFN2 respectively. Only relevant restriction sites are indicated. E, *Eco* RV; X, *Xmn* I; Bt, *Bst* EII; Bg, *Bgl* II; S, *Ssp* I; Hc, *Hinc* II; Bs, *Bst* XI; H, *Hind* III; A, *Apa* I; K, *Kpn* I; Xb, *Xba* I; C, *Cla* I; B, *Bam* HI. B. Southern blot analysis of DNA from wild-type strain, WT, SFN1 and SFN2 cultivated for several generations in BG11 medium (+ and − indicate the two orientations of the cassettes inserted). Chromosomal DNA was digested with *Bst* XI. The probes used were the whole *gltB* and *gltS* genes for the hybridizations of SFN1+/− and SFN2+/− respectively.

hybridization with the two probes used (Fig. 2A and B), suggesting a high homology between them.

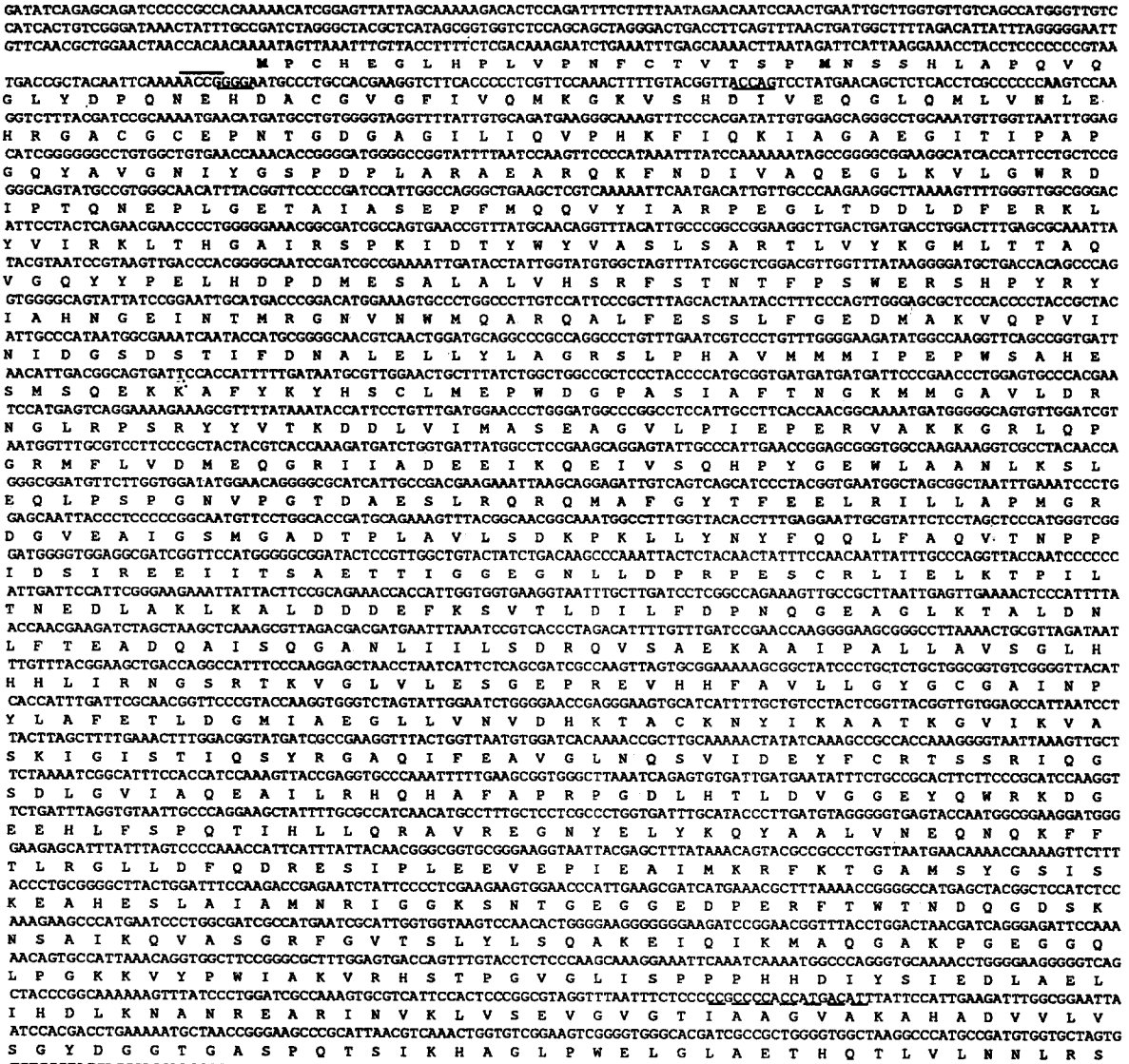
Based on the restriction analysis of pFN3 and pFN4, the 6.3 kb *Hind* III (Fig. 2A, lane 1) and 9.3 kb *Cla* I (Fig. 2B, lane 1) fragments, which could contain the full *gltB* and *gltS* genes, were cloned as indicated above, by screening colonies in *E. coli* HB101, using the probes I and II, respectively, indicated in Fig. 3A. Out of the 256 clones tested for the *Hind* III fragment and 477 for the *Cla* I fragment, two (plasmids pFN5 and pFN6) and fourteen (plasmids pFN7 to pFN21) respectively were positives.

The restriction map of pFN5 overlaps the pFN3 insert and extends about 4 kb upstream including the *gltB* gene, while the restriction map of pFN7 overlaps the pFN4 insert, extending upstream about 7 kb, containing *gltS* gene (Fig. 3A).

Sequence analysis of gltB

The nucleotide sequence of virtually the whole insert contained in plasmid pFN5 (5214 bp) was determined on both strands. One open reading frame of 4650 bp encoding a 1550 residue polypeptide was identified. Two putative ATG start codons are present preceded by potential ribosome binding sites (Fig. 4). A search of the SWISS-PROT and EMBL/GenBank databases revealed significant sequence identity of *Synechocystis* Fd-GOGAT GltB with all the glutamate synthases sequenced so far. As shown in Figs. 5 and 6, a comparative analysis with the GOGATs sequences reveals that the identity of the GltB amino acid sequence is higher with the NADH-GOGAT from alfalfa (55.2%) than with the higher plants and red alga Fd-GOGAT (42–44%) and with bacterial NADPH-GOGAT (41–45%), suggesting that, although they are relatively well conserved along the evolutionary scale, alfalfa NADH-GOGAT seems to be more related to the protein from cyanobacteria than its ferre-

Fig. 4. Nucleotide and deduced amino acid sequences of the gene coding for GltB of *Synechocystis*. Amino acid residues are numbered consecutively. The two putative initial methionines are in bold. Putative ribosome-binding sites are under or upperlined (———). The two oligonucleotides used for PCR amplification are dashed underlined (– – – – –). Arrows denote a potential stem-loop structure in the 3′ downstream region.




GLUTAMINE AMIDE TRANSFERASE DOMAIN
Maturation site
Fd-Spinach
Fd-Maize
Fd-Antitha
NADH-alfalf
Fd-Synechoc
NADPH-Azosp
NADPH-E.col
Fd-Spinach
Fd-Maize
Fd-Antitha
NADH-Alfal
Fd-Synechoc
NADPH-Azosp
NADPH-E col
Fd-Spinach
Fd-Maize
Fd-Antitha
NADH-Alfal
Fd-Synechoc
NADPH-Azosp
NADPH-E col
Fd-Spinach
Fd-Maize
Fd-Antitha
NADH-Alfal
Fd-Synechoc
NADPH-Azosp
NADPH-E col
Fd-Spinach
Fd-Maize
Fd-Antitha
NADH-Alfal
Fd-Synechoc
NADPH-Azosp
NADPH-E col

Fig. 5. Comparison of amino acid sequences of glutamate synthases. The *Synechocystis* GltB amino acid sequence was compared to the glutamate synthase sequences available, with the PILEUP program [39], using the parameters indicated in Materials and methods and improved by manual fitting. Glutamine amidotransferase domain and the putative maturation site are upperlined and underlined respectively. The FMN domain is also upperlined and the 3Fe-4S cluster is shadowed. The aspartic acid and the lysine/arginine residues involved in the binding of the ribityl chain of FMN are marked by ▲. The 3 cysteines of the iron-sulfur cluster are denoted by △. Asterisks indicate identical amino acid residues in all the sequences. The glutamate synthase sequences were obtained from the EMBL/GenBank and were Fd-GOGAT from maize [45], spinach [38], the red alga *Antithamnion* [52], NADH-GOGAT from alfalfa [19], and the NADPH-GOGATs from *A. brasilense* [42] and *E. coli* [40].

doxin counterpart or the bacterial NADPH-GOGAT (Fig. 6). It is worth noting that all glutamate synthases show a basal identity, about 41 to 45% (Fig. 6). In the case of eukaryotic Fd-GOGATs, this identity increases to 83% when higher-plant Fd-GOGATs are compared between them, being 56% when comparison was done with the Fd-GOGAT of the red alga *Antithamnion* (Fig. 6).

Identification of functional domains in Synechocystis *glutamate synthase* (gltB *gene)*

All the glutamate synthases purified from different sources, including bacterial (NADPH-GOGAT) and plant (ferredoxin and NAD(P)H-GOGAT) types, contain several prosthetic groups, such as flavins (FMN and FAD) and iron-sulfur centres (4Fe-4S or 3Fe-4S). The increase in the number of sequences available makes it now possible to search for amino acid sequence similarities in the different domains, especially flavin-binding sites and the 3Fe-4S centre, which have been found in all the glutamate synthases analysed. In our case, the GltB amino acid sequence was compared with the corresponding amino acid sequences from bacterial NADPH-GOGAT α-subunit (*gltB* gene), the photosynthetic eukaryotes Fd-GOGAT (*gltS* gene) and NADH-GOGAT. The glutamine amidotransferase domain includes a region (residues 43–102) of *Synechocystis* GltB protein, in the N-terminus, which was found to be similar to the same region in other GOGATs and also to the glutamine amidotransferase domain of *purF* [33, 34] (Fig. 5) while a second region (residues 250–260) was more similar to the same domain in trpG-type amidotransferases [33] (not shown). A cysteine, Cys-43, is absolutely conserved in all GOGATs sequenced so far, and implicated in the release of the glutamine amide group [33, 34, 53]; Cys-43 seems to be also a specific site recognized by a protease to give a mature α-subunit in bacteria [40] and also a mature Fd-GOGAT in maize, independently of the chloroplast transit peptide sequence upstream of it [45].

A second strongly conserved region corresponds to the 3Fe-4S cluster, although in bacteria another iron-sulfur cluster has been found but only in the NADPH-GOGAT small subunit [54]. The 3Fe-4S cluster comprised a cysteine rich region in *Synechocystis gltB* (Cys-1150, Cys-1156, and Cys-1161), which aligns with similar cysteines in the other GOGAT amino acid sequences [27] (Fig. 5). A similar spacing of the cysteine cluster (CX_5CX_4C) was also reported for the enzymes fumarate reductase and succinate dehydrogenase that also contain the 3Fe-4S cluster [25, 30], supporting the assignment of these cysteines to the

	1	2	3	4	5	6	7
1 Fd-*Synechocystis*	-	55.2	41.7	43.7	41.9	45.0	41.2
2 NADH-Alfalfa		-	43.1	44.1	41.9	43.9	42.5
3 Fd-Spinach			-	83.6	55.2	43.8	42.6
4 Fd-Maize				-	55.8	43.9	42.5
5 Fd-*Antithamnion*					-	42.2	41.1
6 NADPH-*A. brasilense*						-	42.5
7 NADPH-*E. coli*							-

Fig. 6. Protein sequence identity among glutamate synthases. Identities are based on the alignment showed in Fig. 5, from the amino acid 97 to 1602 of the maize sequence. The identity between each two glutamate synthases was calculated respect to the shortest sequence.

above-mentioned iron-sulfur cluster. In addition, other cysteines, such as Cys-1415 of *Synechocystis* protein, are also conserved in all GOGATs, what could indicate a role of the carboxy-terminal portion of the protein in the catalytic function or in the folding of glutamate synthases.

A region of the *Synechocystis* GltB amino acid sequence, which includes the residues 1013–1145 (Fig. 5), is strongly conserved in all GOGATs (identity higher than 60%). In particular 12 glycine residues are fully conserved, which suggests a characteristic conformation of the protein in this region. In fact, glycine-rich regions have been demonstrated to form β-α-β secondary structures in other flavoproteins [26, 55]. One FMN binding domain of the yeast flavocytochrome b_2 shows many residues identical to this region of GltB and also with the other GOGATs sequences. Two critical amino acid residues, Asp-1118, corresponding to flavocytochrome Asp-419, and Lys-1122, corresponding to Arg-423, which interact with the ribityl side-chain of FMN in the flavocytochrome b_2 reductase of yeast are conserved as well [10]. The fact that many other residues are also identical suggests that the structure needed to attach the FMN molecule requires some other amino acids to allocate this flavin correctly for its concourse in the electron transfer chain from ferredoxin to the reductive amination of 2-oxoglutarate. On the other hand, a putative region for the FAD binding, which seems to be lacking in the Fd-GOGAT of the cyanobacterium *Synechococcus* PCC 6301 [31] but present in other Fd-GOGATs [18, 22], is localized in the amino acids region 1396–1446 of *Synechocystis* GltB. If FAD is absent in other cyanobacterial glutamate synthases need to be studied. The purification and analysis of *Synechocystis* GltB protein would help to confirm this fact.

Insertional mutagenesis of Synechocystis gltB *and* gltS *genes*

In order to generate *Synechocystis* mutant strains lacking the proteins coded by *gltB* and *gltS* genes, we constructed the plasmids pFN10+/− and pFN11+/− by interrupting the *gltB* and *gltS* genes with a chloramphenicol (Cm^R) or a kanamycin (Km^R) resistance cassette respectively, in both

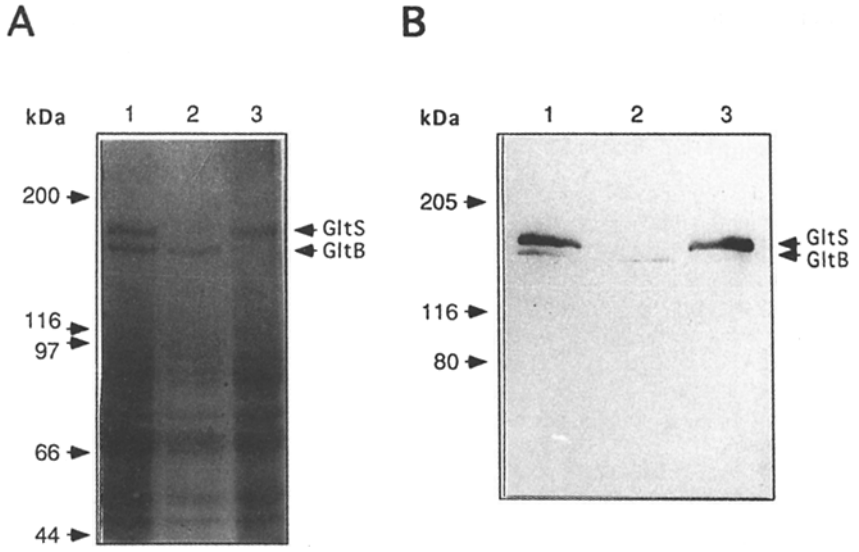


Fig. 7. Detection of *Synechocystis* Fd-GOGAT polypeptides. A. Fd-GOGATs partially purified from *Synechocystis* wild-type (lane 1), SFN2+ (lane 2) and SFN1+ (lane 3) were separated by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue. B. Western blot of the result of SDS-polyacrylamide gel electrotransferred to a nitrocellulose membrane and incubated with antibodies against *Synechococcus* 6301 Fd-GOGAT (lanes as in A). 60 μg protein were loaded in each lane.

orientations (Fig. 3A). Four different transformations of WT *Synechocystis* were performed, using each of the four plasmids aforementioned. After 5–6 days, kanamycin and chloramphenicol-resistant transformants were selected at a similar frequency in BG11 medium containing nitrate as the nitrogen source. After several segregation rounds in the same medium, by increasing the amount of the corresponding antibiotics, total DNA were isolated from the strains SFN1+, SFN1–) (*gltB* mutants), SFN2+ and SFN2– (*gltS* mutants) and from the WT strain. Analysis by Southern blot using the complete *gltB* or *gltS* genes as probes, to avoid the cross-hybridization, showed that SFN1+/– and SFN2+/– were homozygous for the interrupted *gltB* and *gltS* genes respectively (Fig. 3B). As all the mutants were capable to grow in the culture medium used, we deduced that both genes were expressed in *Synechocystis* or that glutamate biosynthesis could be supported by the activity of the NADP-glutamate dehydrogenase [17] in at least one of the mutants. To demonstrate that *gltB* and *gltS* genes are actively expressed and that the two corresponding proteins are functional, we determined glutamate synthase activity in the strains SFN1+ and SFN2+ and WT *Synechocystis*. It was noted that all the mutants grew at the same rate as WT (not shown). As indicated in Table 1, Fd-GOGAT activity of the mutant SFN1+ was about 75% of the activity determined in the WT strain, while SFN2 + activity was about 50%. No NAD(P)H-GOGAT activity was detected in any case, confirming that each gene codes for a different Fd-GOGAT and that the enzyme coded by the *gltS* gene seems to be more active than the one coded by *gltB* (Table 1). These results also indicate that both genes are nonessential if one of them is expressed, since trying to obtain a *gltS*, *gltB* double mutant failed in all the growth conditions used (data not shown).

Table 1. Fd-GOGAT activity of wild-type, SFN1+ and SFN2+ strains of *Synechocystis*. Fd-GOGAT activity was determined in crude extracts obtained from cells grown in BG11 medium with nitrate as the nitrogen source. Initial reaction rates were determined as described in Materials and methods. The values represent averages of three measurements ± standard error. Glutamate synthase activities dependent on NADPH or NADH were negligible in all the strains tested.

Strain	Fd-GOGAT activity (mU/mg protein)	(%)
PCC 6803	54.42 ± 3.7	100
SFN1+ (*gltB* mutant)	41.93 ± 5.8	77
SFN2+ (*gltS* mutant)	25.40 ± 3.6	47

Identification and characterization of the two Fd-GOGATs

To identify the two polypeptides coded by *gltB* and *gltS* genes, we addressed the partial purification of both proteins, using WT, SFN1+ and SFN2+ *Synechocystis* strains. Although the two Fd-GOGATs could be partially purified using WT *Synechocystis*, the mutant strains are of interest since GltS and GltB can be independently isolated from SFN1+ and SFN2+ respectively. The elution on chromatography resulted in a five-fold purification with a recovery of 75–80%. As shown in Fig. 7A, two polypeptides of molecular mass of 170 and 180 kDa appeared in a 6.5% SDS-PAGE as a result of the partial purification of WT strain glutamate synthases (Fig. 7A, lane 1), while the mutant strain SFN1+ gave only the 180 kDa polypeptide (GltS) and SFN2+ only the 170 kDa polypeptide (GltB) (Fig. 7A, lanes 3 and 2). When western blotting was performed, using antibodies against *Synechococcus* PCC 6301 Fd-GOGAT, only the polypeptides indicated above cross-reacted (Fig. 7B), confirming the existence of the two Fd-GOGAT, clearly different in size, in the WT *Synechocystis* strain and encoded by different genes that could be independently inactivated.

Conclusions

The utilization of PCR techniques has allowed us to clone two different genes encoding ferredoxin-dependent glutamate synthases from the cyanobacterium *Synechocystis* sp. PCC 6803. One of

them, *gltB*, has been fully sequenced and its comparison with other glutamate synthases indicates significant identities with all of them. The fact that the higher identity of GltB was found with the plant NADH-GOGAT and that all GOGATs so far known have a basal identity about 42%, suggest a common origin of the different types of GOGATs. The recent evidence that Fd-GOGAT is encoded in the chloroplast of the red algae *Antithamnion* sp. and *Porphyra purpurea* [43, 52] supports the view that glutamate synthases were initially plastid encoded in photosynthetic eukaryotes and during evolution their genes were transferred to the nucleus. In addition, our data strongly suggest that both genes are functional in this cyanobacterium.

Further studies would be required to define the specific function of each Fd-GOGAT and if the existence of two different genes is a peculiarity of *Synechocystis* or a characteristic of cyanobacteria. In addition, the complete sequence of *gltS*, now in progress, will allow to carry out a phylogenetic analysis of the cyanobacterial Fd-GOGATs.

Acknowledgements

This work was supported by a grant of the Dirección General de Investigación Científica y Técnica of Spain (PB91-0127) and by Junta de Andalucía (group 3247). Francisco Navarro is the recipient of a predoctoral fellowship from the Fundación Cámara-Universidad de Sevilla. We thank Dr Silvia Marqués and Dr Jean Vidal for antibody preparation.

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