

## Role of GOGAT in carbon and nitrogen partitioning in *Rhizobium etli*

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**The isolation and characterization of a *Rhizobium etli* glutamate auxotroph, TAD12, harbouring a single Tn5 insertion, is reported. This mutant produced no detectable glutamate synthase (GOGAT) activity. The cloning and physical characterization of a 7.2 kb fragment of *R. etli* DNA harbouring the structural genes *gltB* and *gltD* encoding the two GOGAT subunits GltB and GltD is also reported. In comparison with the wild-type strain (CFN42), the GOGAT mutant strain utilized less succinate and glutamate and grew less with this and other amino acids as nitrogen source. *R. etli* assimilates ammonium by the glutamine synthetase (GS)-GOGAT pathway and a GOGAT mutant prevents the cycling of glutamine by this pathway, something that impairs nitrogen and carbon metabolism and explains the decrease in the amino-nitrogen during exponential growth, with glutamate as nitrogen source. GOGAT activity also has a role in ammonium turnover and in the synthesis of amino acids and proteins, processes that are necessary to sustain cell viability in non-growing conditions. The assimilation of ammonium is important during symbiosis and glutamate constitutes 20–40% of the total amino-nitrogen. In symbiosis, the blockage of ammonium assimilation by a GOGAT mutation significantly decreases the amino-nitrogen pool of the bacteroids and may explain why more N<sub>2</sub> is fixed in ammonium, excreted to the plant cell, transported to the leaves and stored in the seeds.**

**Keywords:** nitrogen assimilation, carbon metabolism, glutamine turnover, nitrogen fixation, glutamate auxotroph

### INTRODUCTION

Virtually all micro-organisms that fix atmospheric nitrogen in free life assimilate ammonium through the glutamine synthetase (GS)-glutamate synthase (GOGAT) pathway (Kanamori *et al.*, 1989). The GS-GOGAT pathway has a higher affinity for ammonium than the alternative pathway composed of glutamate dehydrogenase (GDH)-glutamine synthetase (GS). The use of the GS-GOGAT pathway guarantees the efficient

assimilation of the limited amounts of ammonium formed under diazotrophic conditions.

We have shown previously that in *Rhizobium etli*, as in many other *Rhizobium* species, ammonium is assimilated exclusively by the GS-GOGAT pathway (Bravo & Mora, 1988; Kondorosi *et al.*, 1977; Ludwig, 1980; O'Gara *et al.*, 1984; Osburne & Signer, 1980). Accordingly, all the reported *Rhizobium* strains impaired in GOGAT activity are glutamate auxotrophs (Kondorosi *et al.*, 1977; Ludwig, 1980; O'Gara *et al.*, 1984). The GS-GOGAT pathway is also necessary for efficient succinate consumption in several isolates of *Rhizobium* and *Sinorhizobium* (Encarnación *et al.*, 1998). In these strains, succinate utilization is abated when the three GS activities are decreased, demonstrating a significant linkage between nitrogen and carbon metabolism through cycling of the glutamine pool (Encarnación *et al.*, 1998; Mora, 1990).

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**Abbreviations:** GDH, glutamate dehydrogenase; GOGAT, glutamate synthase; GS, glutamine synthetase; MM, minimal media; MSO, methionine sulfone.

The GenBank accession number for the sequence reported in this paper is AF107264.

Although it has been accepted that *Rhizobium* bacteroids do not assimilate ammonium during symbiosis (Kurz *et al.*, 1975), there is abundant evidence indicating that bacteroids synthesize different nitrogen-containing metabolites during symbiosis (Salminen & Streeter, 1987; Streeter, 1987; Kouchi *et al.*, 1991; Rastogi & Watson, 1991). Furthermore, significant GS and GOGAT activities have been detected in bacteroids isolated from nodules formed on different legume species, including bean plants inoculated with *Rhizobium leguminosarum* bv. *phaseoli* (Brown & Dilworth, 1975; Durán *et al.*, 1995; Moreno *et al.*, 1991). Although several *Rhizobium* GOGAT mutants have been reported, none of them has been thoroughly characterized (Hilgert *et al.*, 1987; Kondorosi *et al.*, 1977; Lewis *et al.*, 1990). The isolation and study of a *R. etli* GOGAT mutant would improve our knowledge of free-living and symbiotic nitrogen and carbon metabolism.

We are interested in the rational modification of the ammonium assimilation pathway in *Rhizobium* to assess and, if possible, enhance its symbiotic performance. In this regard, we have demonstrated that increasing the internal nitrogen content of *R. etli* by the constitutive expression of a heterologous GDH activity during nodule establishment leads to a reduction of *nod* gene expression and, as a consequence, impairs nodule formation (Mendoza *et al.*, 1995). This impairment is prevented if *gdhA* expression is placed under the control of a NifA-dependent promoter and the induction of GDH activity is delayed until nodules are formed (Mendoza *et al.*, 1998). The controlled expression of GDH in bacteroids modifies nitrogen partitioning and leads to the newly synthesized ammonium being preferentially incorporated into the amino acid pools, instead of being exported to the plant cells. As a consequence, the amount of fixed nitrogen transported to the leaves is significantly decreased. Furthermore, the bacteroid nitrogenase activity is drastically affected, resulting in a lowered bean-plant yield (Mendoza *et al.*, 1998).

The results described above suggest that the lowering of the basal nitrogen metabolism in *Rhizobium* bacteroids by obstructing the GS-GOGAT pathway may stimulate nitrogenase activity and increase the nitrogen supply to the plant. In the present work, we present the cloning and physical characterization of the *R. etli* CFN42 *glt* locus, the isolation of a GOGAT mutant, and its physiological characterization in free life and during symbiosis.

## METHODS

**Bacterial strains, plasmids and media.** The bacterial strains and plasmids used in this work are listed in Table 1. *Escherichia coli* strains were grown at 37 °C on LB complex medium (Sambrook *et al.*, 1989). *R. etli* strains were grown at 30 °C on PY complex medium or on minimal medium (MM) containing 10 mM succinate and 10 mM NH<sub>4</sub>Cl, as carbon and nitrogen sources, unless otherwise stated (Encarnación

*et al.*, 1995). Antibiotics were added at the following final concentrations (µg ml<sup>-1</sup>): kanamycin, 30; nalidixic acid, 20; carbenicillin, 100; and tetracycline, 10; streptomycin, 200. Conjugation was carried on solid agar using cultures grown to late-exponential phase in rich media (PY or LB). Aliquots (0.2 ml) of donors, recipient and helper strains were plated on PY medium and incubated at 30 °C for 48 h. Mating mixtures were streaked on selective medium.

**Growth conditions.** *R. etli* cultures were grown overnight on PY medium. Cells were collected by centrifugation at 6000 g, washed with sterile MM and concentrated 100-fold. Fresh MM containing the desired carbon and nitrogen source was inoculated with this suspension to an initial OD<sub>540</sub> of 0.05. Cultures were grown with shaking at 200 r.p.m. for 24 h at 30 °C. At different times 1 ml aliquots were taken from the culture medium, collected by centrifugation (6000 g) and resuspended in 1 ml 5% (w/v) trichloroacetic acid (TCA). Protein determination was done by the Lowry method.

**Determination of GOGAT activity.** Exponentially growing cells of *R. etli* from 150 ml of 12 h MM cultures were obtained by centrifugation at 12000 g for 10 min. The cell pellet was resuspended in 1.5 ml breaking solution [0.1 M KCl and 0.5% (v/v) 2-mercaptoethanol, adjusted to pH 7.6], disrupted by sonication and centrifuged for 3 min at 12000 g at 4 °C. GOGAT activity was determined spectrophotometrically by following the glutamine-dependent oxidation of NADPH at 340 nm. The reaction mixture contained 50 mM HEPES buffer pH 8.5, 1% (v/v) 2-mercaptoethanol, 3.65 mM glutamine, 3 mM 2-oxoglutarate, 0.2 mM NADPH and 0.1 ml cell-free extract in a final volume of 1 ml. Assays were run at room temperature in a Beckman DU7500 spectrophotometer. Specific activities are reported as nmol NADPH oxidized min<sup>-1</sup> (mg protein)<sup>-1</sup>. To inhibit GOGAT specific activity, 10 mM of methionine sulfone (MSO) was used in the reaction mixture (Bravo & Mora, 1988).

**DNA manipulations, blotting and sequencing.** All routine DNA manipulations, Southern blotting, hybridizations, ligations and *E. coli* transformations were performed following standard procedures (Sambrook *et al.*, 1989). DNA fragments were purified from agarose gels using a GeneClean Kit (Bio101). High-stringency hybridizations using the internal 5.2 kb *Sall* fragment from plasmid pHB10 were performed with the Rapid-Hyb Buffer following the product instructions (Amersham Life Science). Fragments harbouring the *R. etli* *gltBD* genes from cosmid pC12 were cloned into the vector pBluescript SK (see Table 1 and Fig. 1). Automated nucleotide sequencing of these fragments was performed at MediGene GmbH. The *gltBD* gene sequences were determined two or more times on both strands. DNA analysis, such as BLAST, was performed using the National Center for Biotechnology Information Server ([www.ncbi.nih.gov/](http://www.ncbi.nih.gov/)). The multiple alignments were performed using the CLUSTAL W Interactive Sequence Multiple Alignment software at the European Bioinformatics Institute Server ([www2.ebi.ac.uk/clustalw/](http://www2.ebi.ac.uk/clustalw/)). The *gltBD* gene sequences have been deposited in GenBank and assigned the accession number AF107264.

**Mutagenesis of the *glt* locus.** The Tn5-containing suicide plasmid pSUP5011 was conjugated into *R. etli* CFN42 and 12000 random Tn5 insertions were selected in PY plus kanamycin, nalidixic acid and streptomycin. All colonies were replica-plated on MM-glutamate or MM-ammonium and those exhibiting glutamate auxotrophy were segregated and tested in liquid MM. Two glutamate auxotrophs, strains TAD11 and TAD12, were isolated and further characterized.

**Table 1.** Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties	Source or reference
<b>Strains</b>		
<i>E. coli</i>		
HB101	F <sup>-</sup> Δ( <i>gpt-proA</i> ) <i>recA13 lacY1 rpsL20</i> Sm <sup>r</sup>	Boyer & Roulland-Dussoix (1969)
<i>R. etli</i>		
CFN42	Wild-type strain Nal <sup>r</sup>	Quinto <i>et al.</i> (1982)
CE3	Derivative of CFN42 Nal <sup>r</sup> Sm <sup>r</sup>	Noel <i>et al.</i> (1984)
TAD12	<i>gltB</i> :: Tn5 derivative of CE3 Sm <sup>r</sup> Nal <sup>r</sup> Km <sup>r</sup>	This work
<b>Plasmids</b>		
pSUP5011	Tn5- <i>mob</i> in pBR325 Km <sup>r</sup> Cb <sup>r</sup>	Simon <i>et al.</i> (1983)
pRK2013	Mating helper, RK2, <i>mob</i> <sup>+</sup> <i>tra</i> <sup>+</sup> Km <sup>r</sup>	Figurski & Helinski (1979)
pLAFR1	Wide-host-range cloning cosmid <i>mob</i> <sup>+</sup> <i>tra</i> <sup>-</sup> IncP Tc <sup>r</sup>	Friedman <i>et al.</i> (1982)
pBluescript SK	Cloning vector Cb <sup>r</sup>	Stratagene
pHB10	<i>A. sesbaniae gltBD</i> locus in pLAFR1 Tc <sup>r</sup>	Hilgert <i>et al.</i> (1987)
pT12	<i>gltB</i> :: Tn5 from strain TAD12 in pSK Km <sup>r</sup> Cb <sup>r</sup>	This work
pC12	<i>R. etli gltBD</i> locus in pLAFR1 Tc <sup>r</sup> 20 kb insert	This work
pAD1	1.8 kb <i>EcoRI</i> fragment from pC12 in pSK Cb <sup>r</sup>	This work
pAD4	1.6 kb <i>EcoRI</i> fragment from pC12 in pSK Cb <sup>r</sup>	This work
pAD8	0.8 kb <i>EcoRI</i> fragment from pC12 in pSK Cb <sup>r</sup>	This work
pAD3	1.6 kb <i>EcoRI</i> fragment from pC12 in pSK Cb <sup>r</sup>	This work
pAD6	1.0 kb <i>EcoRI</i> fragment from pC12 in pSK Cb <sup>r</sup>	This work

**Determination of ureides.** Groups of five bean (*Phaseolus vulgaris* cv. Negro Jamapa) plants were decapitated 1 cm above the crown at 25 and 32 d post-inoculation. A 3-cm-long rubber tube was attached to collect the xylem sap and the samples were pooled. Ureide content was determined by a colorimetric assay (Vogels & Van der Drift, 1970).

**Isolation of bean bacteroids.** To determine the symbiotic expression of GOGAT, bacteroids were isolated from fresh nodules (5 g) of 3-week-old bean plants following the procedure of Michiels *et al.* (1998). For this, the nodules were detached from the roots of several plants inoculated with the same strain, crushed on ice in 15 ml plastic tubes in the presence of 0.5 g polyvinylpyrrolidone and 2 ml Mg-phosphate buffer (2.5 mM MgCl<sub>2</sub>, 50 mM potassium phosphate, pH 6.8). Next, the paste was diluted with 10 ml ice-cold Mg-phosphate buffer. The tubes were centrifuged at 120 g for 2 min to remove plant cell material. This step was repeated with the supernatant. Bacteroids were finally concentrated at 4 °C by centrifugation at 4500 g for 5 min and resuspended in 1 ml Mg-phosphate buffer. Bacteroids were immediately assayed for GOGAT activity and their amino acid content.

**Determination of free amino acids and organic acids.** Cell and medium samples (150 and 20 ml, respectively) were withdrawn from cultures as described previously (Bravo &

Mora, 1988). The procedure for concentration and re-suspension of the medium samples for determination of organic acids and amino acids was as described previously (Encarnación *et al.*, 1995). Organic acids from cell and medium samples were separated and quantified by injecting 10 and 50 µl, respectively, into a Waters model 510 chromatograph as described previously (Encarnación *et al.*, 1995). Amino acids were quantified fluorimetrically using the pre-column derivation technique with 9-fluoroenylmethyl-chloroformate and a Nova-Pack C18 column (Waters; 3.9 i.d. × 150 mm) (Cevallos *et al.*, 1996). Amino acid separation was performed with a 20–73% elution gradient of solvents A and B (solvent A, 0.05 M sodium acetate, pH 3.5–3.8 adjusted with acetic acid; solvent B, acetonitrile) at a flow rate of 1 ml min<sup>-1</sup> at 45 °C. Amino acids were detected with a 470 AC fluorometer (Waters) set at excitation and emission wavelengths of 254 and 313 nm, respectively, and equipped with a G475 Hg lamp (Waters). Amino acid concentrations were expressed as nmol amino acid (mg protein)<sup>-1</sup>. Amino nitrogen content was obtained by adding the concentration of nmol of each amino acid per sample, except for glutamine, lysine and asparagine, which were counted as two, and arginine, counted as three, and the results expressed in nmol amino nitrogen (mg protein)<sup>-1</sup>.

**Extracellular ammonium determination.** Samples (10 ml) of

medium were collected by filtration through membrane filters (Millipore, type HA, 0.45 µm) and after addition of 0.1 ml 10 M NaOH, measurements of extracellular ammonium concentrations were performed with an Orion electrode as described elsewhere (Espin *et al.*, 1979).

**Determination of the symbiotic phenotype of the GOGAT mutant.** *P. vulgaris* cv. Negro Jamapa seedlings were surface sterilized and germinated as reported previously (Bravo & Mora, 1988). *R. etli* strains used as inoculum were grown overnight in PY medium, washed twice with 0.8% NaCl and diluted to an OD<sub>540</sub> of 0.05. Germinated seedlings were planted in groups of five in pots containing sterile vermiculite as support, and each seedling was inoculated with 1 ml bacterial suspension (approx. 10<sup>7</sup> cells). Each experiment included non-inoculated control plants with and without added nitrogen which were sampled at the same times as the inoculated treatments. When any of the non-inoculated control plants were nodulated, the whole experiment was discarded. Greenhouse conditions were: temperature 22–28 °C and relative humidity 50–60%. Groups of eight plants grown under each condition were harvested at 18, 25 and 32 d post-inoculation and their nodule dry weight, nitrogenase activity, total plant dry weight and nitrogen content were determined. The identity of bacteria isolated from nodules was verified by their antibiotic resistance pattern. Nitrogenase specific activity measured as acetylene reduction [µmol ethylene generated h<sup>-1</sup> (g nodule dry weight)<sup>-1</sup>] was determined by incubating the detached root with 1/80 (v/v) acetylene and determining ethylene production with a Varian 3300 gas chromatograph. Total nitrogen content of powdered dry plants or seeds was determined in a nitrogen analyser model ANTEK 7000 and is reported as mg nitrogen (mg dry weight)<sup>-1</sup>. Bean seeds were harvested at 115–120 d post-inoculation.

**Reproducibility of results.** The experiments reported were carried out at least twice; representative results are shown in some experiments and in others, means and standard deviations were calculated according to Steel & Torrie (1980).

## RESULTS

### Isolation of the *R. etli* *glt* locus

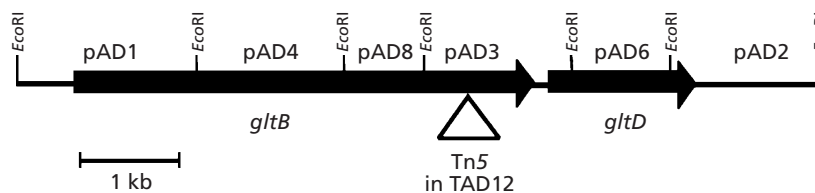
Two *R. etli* glutamate auxotrophs were obtained by screening a random Tn5 insertion library of strain CFN42. In one mutant, strain TAD12, the Tn5 insertion was identified inside the *gltB* structural gene by heterologous hybridization against the *Azorhizobium sesbaniae* *gltBD* genes (data not shown). The Tn5-harboured *EcoRI* fragment from strain TAD12 was cloned and sequenced. According to the nucleotide sequences flanking the insertion, the transposon lies

between nucleotides 1230 and 1231 of the *gltB* gene of *R. etli*. These results allowed us to deduce that the Tn5 insertion was in the *gltB* structural gene (Fig. 1). Strain TAD12 could not grow on ammonium as sole nitrogen source in comparison with the wild-type strain CFN42 (2 h mean doubling time). Whilst CFN42 had a GOGAT specific activity of 65.0 (±3.0) nmol NADPH oxidized min<sup>-1</sup> (mg protein)<sup>-1</sup>, the mutant TAD12 contained 0.7 (±0.15) nmol NADPH oxidized min<sup>-1</sup> (mg protein)<sup>-1</sup>, without MSO (an inhibitor of GOGAT activity) in the reaction mixture. The growth of this strain in MM with succinate plus ammonium was restored when the mutant was complemented by plasmid pHB10 harbouring the *A. sesbaniae* *gltBD* genes (data not shown).

To isolate the *R. etli* *glt* genes by growth complementation of strain TAD12, we used a *R. etli* genomic library cloned in the broad-host-range cosmid vector pLAFR1 (Durán *et al.*, 1996). Cosmids from the genomic library maintained in *E. coli* HB101 were conjugated into strain TAD12 and those transconjugants able to grow on MM succinate plus ammonium plates were selected. Two overlapping cosmids were obtained by this method and designated pC11 (25 kb) and pC12 (20 kb). The mutant TAD12 complemented with pC11 or pC12 grew on MM with succinate plus ammonium, but complementation was better with pC12 based on a comparison of growth in liquid MM after 50 h. Mutant TAD12 complemented with pC11 had a GOGAT specific activity of 96.9 (±9.0) nmol NADPH oxidized min<sup>-1</sup> (mg protein)<sup>-1</sup>, whilst in TAD12 complemented with pC12 this activity was 175.4 (±18.0) nmol NADPH oxidized min<sup>-1</sup> (mg protein)<sup>-1</sup>. In comparison, when GOGAT activity in the CFN42 wild-type strain was inhibited with 10 mM MSO in the reaction mixture, the specific activity was 4.5 (±1.4) nmol NADPH oxidized min<sup>-1</sup> (mg protein)<sup>-1</sup>, whilst the TAD12 mutant showed a sixfold lower GOGAT specific activity than under conditions where there was no inhibitor in the reaction (see above). Cosmid pC12 was selected for subsequent work based on its shorter insert and higher GOGAT activity.

### Physical characterization of the *R. etli* *glt* locus

Restriction-hybridization analysis of the 20 kb insert of cosmid pC12 against a 5.2 kb *SalI* fragment of pHB10 carrying the *A. sesbaniae* *gltBD* genes revealed



**Fig. 1.** Physical map of cosmid pC12 harbouring *gltB* and *gltD* GOGAT-encoding genes, showing the position of the Tn5 insertion in mutant TAD12.

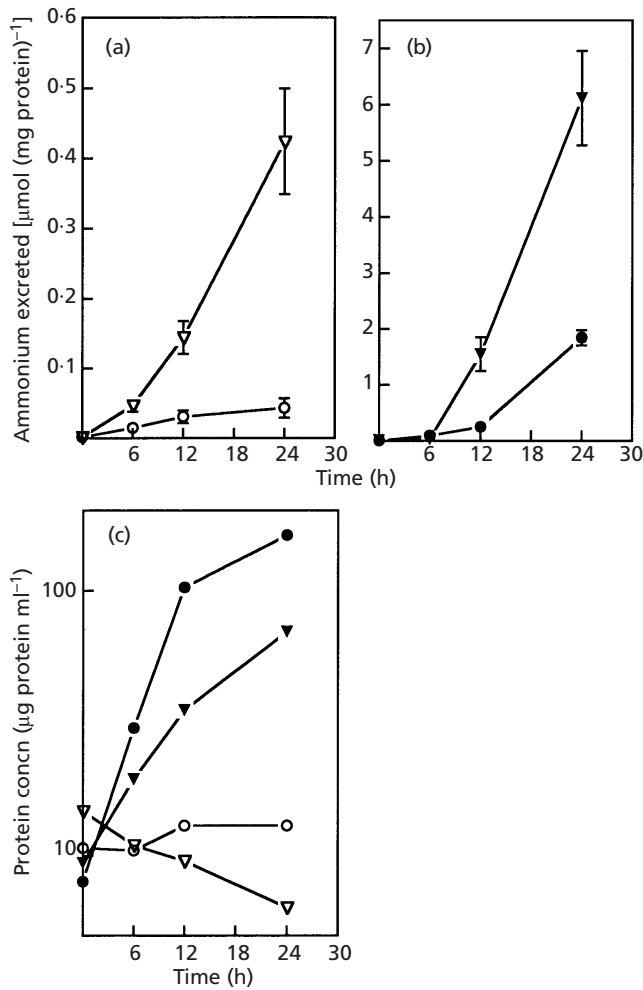
(a)

	1				50
<i>gltB E. coli</i>	mtrkprrrhal	svpvrrgsev	gFpqsIgevh	dmlYdkslEr	DnCGGLIAh
<i>gltB A. bras</i>	mttelnggeq	fvadfranaa	aLttana...	...YnpedEh	DaCGvGFIAa
<i>gltB R. etli</i>	mefdrfaaadtr	atanmsksas	gLpkkqg...	...lYdprnEh	DaCGvGFVAh
Consensus	-----	-----	-L-----	---Y-----E-	D-CG-GFIA-
					..
	51				100
<i>gltB E. coli</i>	ieGepshkVV	rtaihaLarm	qHRGAILADg	ktGDGcG11l	qkPdrFFriv
<i>gltB A. bras</i>	idGkprrrsVV	ekgieaLkav	wHRGAVdADg	ktGDGAGihv	avPqkFFkdh
<i>gltB R. etli</i>	mkGekshqIV	kdglfiLenl	tHRGAVgADp	lmGDGAGilv	qiPdrFFree
Consensus	--G-----VV	-----L---	-HRGAV-AD-	--GDG-G---	--P--FF---
			..	.	.
	1050				1100
<i>gltB E. coli</i>	DLKqVNPkam	IsVKLVSepG	VGTIATGVAK	AyADlItIaG	ydGGTGASPl
<i>gltB A. bras</i>	DLKqINPdak	VtVKLVSRsG	IGTIAaGVAK	AnADiIlIsG	nsGGTGASPl
<i>gltB R. etli</i>	DLKnVNPtsd	VsVKLVSevG	VGTVAAGVAK	ArADhItVaG	fdGGTGASPl
Consensus	DLK-VNP---	V-VKLVs--G	VGtIA-GVAK	A-AD-I-I-G	--GGTGASP-
			*		***
	1101				1150
<i>gltB E. coli</i>	sSvKyAGcPW	ELGLvEtqQa	LvaNgLRhki	rLqvDGGlKT	GvDIikaAiL
<i>gltB A. bras</i>	tSiKfAGlPW	EmGLsEvhQv	LtlNrLRhrv	rLrtDGGlKT	GrDIViaAmL
<i>gltB R. etli</i>	tSfKhaGsPW	EiGLaEtqQt	LvlNgLRsrV	aLqvDGGlKT	GrDVigAll
Consensus	-S-K-AG-PW	E-GL-E--Q-	L--N-LR--V	-L--DGGlKT	G-DII--A-L
				*	*
	1151				1200
<i>gltB E. coli</i>	GAEsFGfgTg	pMVALGckyl	RiChlNnCat	GVatQDdkLR	knhYhG1Pfk
<i>gltB A. bras</i>	GAEeFGigTa	sLIAmGCimv	RqChsNtCpv	GVcvQDdkLR	qk.FvGtPek
<i>gltB R. etli</i>	GADeFGfaTa	pLIAaGCimm	RkChlNtCpv	GVatQDpvLR	kr.FkGtPeh
Consensus	GAE-FG--T-	-LIA-GC---			
		*			
	1151				1200
<i>gltB E. coli</i>	GAEsFGfgTg	pMVALGckyl	RiChlNnCat	GVatQDdkLR	knhYhG1Pfk
<i>gltB A. bras</i>	GAEeFGigTa	sLIAmGCimv	RqChsNtCpv	GVcvQDdkLR	qk.FvGtPek
<i>gltB R. etli</i>	GADeFGfaTa	pLIAaGCimm	RkChlNtCpv	GVatQDpvLR	kr.FkGtPeh
Consensus	GAE-FG--T-	-LIA-GC---	R-CH-N-C--	GV--QD--LR	---F-G-P--
		*	*	*	
	1				50
<i>gltD E. coli</i>	.msqnvvyFi	dlqrvdppkk	PlkiRkiefv	eiyepfSEgq	akaQAdRClS
<i>gltD A. bras</i>	manqrmlgFv	htaqrmpdkr	PaaeRrqdfa	eiyarfSDer	aneQAnRCsq
<i>gltD R. etli</i>	eemgkvtgFl	eidrgvakyq	PasdR.....	...irmSDpe	vqkQAaRCmd
Consensus	-----F-	-----	P---R-----	-----SD--	---QA-RC--
					*
	51				100
<i>gltD E. coli</i>	CGnPYCewk.	.CPVhNyIPn	WlKlanegri	feAaelshqT	NtLPEvcGRV
<i>gltD A. bras</i>	CGvPFCqvh.	.CPVsNnIPd	WlKltsegrl	eeAyevsqaT	NnFPEicGRI
<i>gltD R. etli</i>	CGiPYChgpt	gCPVhNqIPd	WndLvynnnw	eaAiqnlhsT	NnFPEtGRV
Consensus	CG-PYC----	-CPV-N-IP-	W--L-----	--A-----T	N-FPE--GRV
		*	*	*	
	101				150
<i>gltD E. coli</i>	CPqdr1CEgs	Ctlnd.efga	vtignieriI	nDkaFemgwr	pdmSgvkqtg
<i>gltD A. bras</i>	CPqdr1CEgn	Cvieqsthga	vtigsvekyI	nDtaWdggwv	kprtpsrelg
<i>gltD R. etli</i>	CPap..CEea	Ctlndledapv	chpirssrrI	aEggLslrlh	pgrrsrpsit
Consensus	CP----CE--	C-----	-----I	-D-----	-----
	*	*	*		
	301				350
<i>gltD E. coli</i>	gKrVVV1GGG	DTAmDCVrTs	vRQGAKhVtc	ayrrdeenmp	gsrrevknar
<i>gltD A. bras</i>	gKhVVV1GGG	DTAmDCVrTa	iRQGAtsVkc	lyrrdrknmp	gsqrevahae
<i>gltD R. etli</i>	aKhVVVvGGG	DTAsDCVgTa	fRQGAvkVt.	.....	.....
Consensus	-K-VVV-GGG	DTA-DCV-T-	-RQGA-V--	-----	-----
	*****	*****	*****	*****	*****

**Fig. 2.** Amino acid sequence alignment of glutamate synthases with cofactor and substrate binding regions. The amino acid sequence of *R. etli* *gltB* was compared to the glutamine amidotransferase-type PurF domain (a), the FMN-binding domain (b) and the cysteine cluster for the formation of the (3Fe-4S) centre (c). Amino acid sequence of the *gltD* small subunit compared with the cysteine residues (d) and NADPH-binding site (e). The following genes were aligned: *E. coli* *gltBD* accession number M18747 (Oliver *et al.*, 1987) and *A. brasilense* *gltBD* accession number L04300 (Pelanda *et al.*, 1993). The closed circles in (a) denote conserved residues in L-glutamine-dependent amidotransferases (Pelanda *et al.*, 1993); asterisks indicate reported binding sites (Pelanda *et al.*, 1993).

hybridization similarity with five contiguous *EcoRI* fragments covering approximately 7 kb. The cosmid and genomic hybridization patterns were identical (data not shown), reflecting the conservation of the locus organization in cosmid pC12. Although pC12 was subcloned in plasmids pAD1 to pAD9, Table 1 only

shows those subclones harbouring the *glt* locus. Each of the five *EcoRI* fragments detected by Southern hybridization, was subcloned into plasmid pBluescript SK to give plasmids pAD1, pAD3, pAD4, pAD6 and pAD8. These plasmids were used to obtain the *R. etli* *glt* locus nucleotide sequence as described in Methods.



**Fig. 3.** Ammonium excretion by the CFN42 wild-type and TAD12 mutant strains with and without glutamate. *Rhizobium* strains were cultured in MM and aliquots were withdrawn at the indicated times to measure extracellular ammonium as indicated in Methods. (a) Ammonium excretion in MM with succinate (30 mM) without nitrogen source; CFN42 wild-type strain (○) and TAD12 mutant (▽). (b) Ammonium excretion in MM with succinate (30 mM) plus glutamate (10 mM) as nitrogen source; CFN42 wild-type strain (●) and TAD12 mutant (▼). The results are from three independent experiments. (c) Protein concentration of the CFN42 wild-type (○) and TAD12 mutant (▽) strains corresponding to culture conditions as in (a), and CFN42 wild-type (●) and TAD12 mutant (▼) strains corresponding to culture conditions as in (b).

The complete nucleotide sequence of the five plasmids was determined for a total of 7195 bp. Two major open reading frames were identified: ORF1, encoding a protein of 1533 amino acids; and ORF2, encoding a protein of 470 amino acids. The ORFs are separated by 208 nucleotides and each one is preceded by a potential ribosome-binding site. Comparison of the deduced amino acid sequences against the SWISS-PROT database allowed us to identify the ORF1 product as GltB and the ORF2 product as GltD, known as the GOGAT large and small subunits, respectively. The ORF1

**Table 2.** Intracellular glutamine, glutamate and amino-nitrogen content of CFN42 wild-type and TAD12 mutant strains grown with succinate and glutamate

Strains were grown in MM with succinate and glutamate (10 mM each) as the carbon and nitrogen source. Amino acid determinations were done during exponential growth (10 h). Values are expressed in nmol (mg protein)<sup>-1</sup>. Values for CFN42 and TAD12 strains are means ± standard deviation from three and five experiments, respectively. Amino-nitrogen content was obtained by adding the concentration of nmol of each amino acid (Gln, Asp/Ser, Glu, Thr, Gly, Ala, Val, Met, Ile, Leu, Lys and Arg) per sample, except for Gln and Lys, which were counted as two, and Arg, counted as three, and is expressed as nmol (mg protein)<sup>-1</sup>.

Strain	Gln	Glu	Amino-nitrogen
CFN42	4.80 (±2.60)	284.27 (±20.29)*	306.47 (±37.14)*
TAD12	6.22 (±4.47)	93.64 (±96.75)*	123.14 (±86.46)*

\*Indicates that means of samples are significantly different between control and mutant at  $P < 0.05$ .

product is 60% similar to the *Azospirillum brasilense* and *E. coli* GltB proteins, and includes GOGAT characteristic motifs such as the glutamine-amido-transferase-type PurF site between residues 40 and 97 (Fig. 2a), the flavine mononucleotide (FMN)-binding motif between residues 1050 and 1165 (Fig. 2b), and the cysteine cluster involved in the formation of the 3Fe-4S centre between residues 1167 and 1180 (Fig. 2c) (Pelanda *et al.*, 1993). The ORF2 product is 50% similar to the *A. brasilense* and *E. coli* GltD proteins and includes the cysteine residues between residues 48 and 112 (Fig. 2d) and the highly conserved NADPH-binding motif between residues 300 and 330 (Fig. 2e) (Pelanda *et al.*, 1993). The organization of the *R. etli* *glt* locus is similar to that of *E. coli*, with *gltB* upstream of *gltD*, whereas in *A. brasilense* the opposite arrangement occurs (Oliver *et al.*, 1987; Pelanda *et al.*, 1993). The *R. etli* *gltB* and *gltD* genes are 208 nucleotides apart, whereas in *A. brasilense* they are separated by 141 nucleotides, and in *E. coli* by only 12 nucleotides.

#### Free-living phenotype of the *R. etli* GOGAT mutant

It has been reported that the glutamate auxotrophy of *E. coli* (Pahel *et al.*, 1978), *Klebsiella aerogenes* [*K. pneumoniae*] (Brenchley *et al.*, 1973) and *Salmonella typhimurium* (Madonna *et al.*, 1985) GOGAT mutant strains is pleiotropic with respect to other amino acids (Kondorosi *et al.*, 1977; Lewis *et al.*, 1990). Therefore, the TAD12 mutant and the CFN42 wild-type strains were grown on MM with succinate (10 mM) plus different amino acids (glutamate, histidine, arginine, proline and serine; each at 10 mM) as nitrogen source. As expected, the wild-type strain grew with all the amino acids tested, with a mean doubling time of 2.0–2.5 h. The TAD12 mutant grew with glutamate,

**Table 3.** GOGAT activities and glutamate content in bean bacteroids from CFN42 wild-type and TAD12 mutant strains

Bacteroids were isolated as described in Methods. Values are means  $\pm$  standard deviation from three independent experiments.

Strain	Days post-inoculation	GOGAT activity†	Glutamate‡	Total amino-nitrogen§
CFN42	18	36.0 ( $\pm$ 6.0)*	309 ( $\pm$ 80)	1449 ( $\pm$ 440)*
	25	13.0 ( $\pm$ 0.4)*	145 ( $\pm$ 14)	365 ( $\pm$ 136)
	32	21.0 ( $\pm$ 0.7)*	116 ( $\pm$ 28)	350 ( $\pm$ 92)
TAD12	18	1.2 ( $\pm$ 1.0)*	132 ( $\pm$ 117)	416 ( $\pm$ 137)*
	25	3.9 ( $\pm$ 1.4)*	84 ( $\pm$ 36)	254 ( $\pm$ 128)
	32	1.1 ( $\pm$ 0.3)*	63 ( $\pm$ 46)	267 ( $\pm$ 73)

\* Indicates that means of samples are significantly different ( $P < 0.05$ ).

† Expressed as nmol NADPH oxidized  $\text{min}^{-1}$  ( $\text{mg protein}^{-1}$ ).

‡ Expressed as nmol ( $\text{mg protein}^{-1}$ ).

§ See legend of Table 2 for details of amino-nitrogen determination.

histidine, arginine and proline as nitrogen sources, with a mean generation time of 4.0, 2.5, 3.5 and 3.5 h, respectively, but was unable to use serine. This is consistent with serine being directly catabolized to pyruvate and ammonium by serine dehydratase, and the GOGAT mutant being incapable of reassimilating the ammonium produced (Fig. 3). The GOGAT mutant strain consumed 50% and 75% less succinate after 4 and 12 h growth, respectively, than the wild-type strain. Similarly, the GOGAT strain utilized 25% and 50% glutamate at 4 and 12 h growth, respectively.

#### Cellular amino-nitrogen content in the CFN42 wild-type strain and the mutant TAD12

Strain TAD12 lacks its parent strain's sole ammonium assimilation pathway (GS-GOGAT) so an imbalance in the glutamate and amino-nitrogen pools was expected. To determine the extent of this imbalance we measured the intracellular amino acid pools in wild-type and mutant strains during exponential growth on MM with succinate plus glutamate. When strain TAD12 was grown on the latter medium, there was a threefold decrease in the glutamate and in the total amino-nitrogen content compared to the wild-type (Table 2).

#### Amino acid concentration in bean bacteroids of the CFN42 wild-type and the GOGAT mutant TAD12

As expected, bacteroids isolated from nodules formed by the GOGAT mutant lacked GOGAT activity (Table 3). As a consequence of the decreased glutamate synthesis, there was a threefold decrease in the glutamate content in the mutant bacteroids and a significant decrease in the total amino-nitrogen content at 18 d post-inoculation. At 25 and 32 d post-inoculation there was also a decrease in glutamate and in the amino-

nitrogen content in the TAD12 mutant, but the differences in comparison with the wild-type strain were less striking (Table 3).

#### Ammonium excretion by the CFN42 wild-type and TAD12 mutant strains

It has been shown elsewhere that when some *Rhizobium* strains are cultured under carbon-source limiting conditions, the growth rate is reduced and the excess of non-assimilated ammonium is excreted (Encarnación *et al.*, 1998). To evaluate the role of GOGAT upon the fate of ammonium derived from catabolism of an internal or an external source, we estimated the levels of ammonium excreted by the GOGAT mutant during growth on MM containing succinate with and without glutamate. As shown in Fig. 3(a), when cells were cultured in the absence of added nitrogen, significant amounts of ammonium were excreted by the GOGAT mutant but not by the wild-type strain. When grown with glutamate as nitrogen source, the GOGAT mutant began excreting ammonium after 6 h, whereas the wild-type strain did so only after 12 h (Fig. 3b). The excretion by the GOGAT mutant strain after 12 h in glutamate was 10-fold higher than in the absence of nitrogen. It was remarkable that in the absence of an added nitrogen source, 90% of cell viability was lost in the GOGAT mutant at 24 h after incubation in comparison with the wild-type strain (data not shown). This result was the mean of two experiments; moreover, the protein content of the GOGAT mutant decreased, whilst the protein content of the wild-type strain remained constant (Fig. 3c). By contrast, when cells were cultured with glutamate, the growth (protein concentration) of strain CFN42 increased at a normal rate, whereas it was significantly diminished in the GOGAT mutant strain (Fig. 3c; see Discussion).

**Table 4.** Symbiotic phenotype of the CFN42 wild-type and TAD12 mutant strains

Values are means  $\pm$  standard deviation from three experiments with 5–8 plants for each assay and time. Negro Jamapa seedlings were inoculated as described in Methods.

Assay	Days post-inoculation	CFN42	TAD12
Acetylene reduction [ $\mu\text{mol ethylene h}^{-1}$ (g nodule dry weight) $^{-1}$ ]	18	82.47 ( $\pm 7.77$ )*	101.40 ( $\pm 15.21$ )*
	25	29.93 ( $\pm 7.81$ )*	44.57 ( $\pm 12.16$ )*
	32	13.21 ( $\pm 3.12$ )*	19.12 ( $\pm 5.34$ )*
Plant yield (g dry weight per plant) <sup>1</sup>	18	0.28 ( $\pm 0.07$ )	0.26 ( $\pm 0.05$ )
	25	0.43 ( $\pm 0.09$ )	0.41 ( $\pm 0.09$ )
	32	0.95 ( $\pm 0.13$ )	1.01 ( $\pm 0.17$ )
Ureides in xylem sap (mmol)	25	0.083 ( $\pm 0.029$ )	0.135 ( $\pm 0.016$ )
	32	0.100 ( $\pm 0.014$ )	0.112 ( $\pm 0.019$ )
Plant N content [mg N (mg plant dry weight) $^{-1}$ ]	18	0.036 ( $\pm 0.002$ )*	0.043 ( $\pm 0.002$ )*
	25	0.036 ( $\pm 0.002$ )*	0.042 ( $\pm 0.003$ )*
	32	0.038 ( $\pm 0.001$ )*	0.044 ( $\pm 0.002$ )*
Seed N content [mg N (mg powdered seeds) $^{-1}$ ]		0.038 ( $\pm 0.001$ )*	0.044 ( $\pm 0.003$ )*

\* Indicates that means of samples are significantly different ( $P < 0.05$ ).

### Symbiotic phenotype of the *R. etli* GOGAT mutant TAD12

To evaluate the role of the fixed ammonium assimilation during symbiosis, the GOGAT mutant strain was inoculated onto *P. vulgaris* cv. Negro Jamapa seedlings. Plants were collected after 18, 25 and 32 d post-inoculation and their symbiotic properties are presented in Table 4. All the plants inoculated with strain TAD12 nodulated normally, but their nitrogenase activity was 23, 49 and 45% higher at 18, 25 and 32 d post-inoculation, respectively, relative to plants inoculated with the wild-type strain (Table 4). This increase was significantly different ( $P < 0.05$ ). Although the total plant yield (g dry weight per plant) remained similar (Table 4), the nitrogen transport to the leaves, measured as ureides in xylem sap, showed an increase of 62% at 25 d post-inoculation in the mutant strain TAD12 (Table 4), in comparison with the wild-type strain CFN42. After 32 d post-inoculation this increase was only 12% in the mutant strain compared with the wild-type (Table 4). As a consequence, the plant nitrogen content of plants [mg N (mg plant dry weight) $^{-1}$ ] inoculated with strain TAD12 increased 18%  $\pm$  2% (Table 4), and the seed nitrogen content 17% (Table 4), which was significant at  $P < 0.05$ .

### DISCUSSION

In this work we describe the isolation and characterization of a *R. etli* glutamate auxotroph, strain TAD12, harbouring a single Tn5 insertion. This mutant pro-

duced no detectable GOGAT activity and had a moderate assimilation-minus ( $\text{Asm}^-$ ) phenotype as discussed below. We also describe the cloning and physical characterization of a 7.2 kb fragment of *R. etli* DNA harbouring the structural genes *gltB* and *gltD* encoding the two glutamate synthase subunits, GltB and GltD.

GOGAT mutants in *Sinorhizobium meliloti* (Lewis *et al.*, 1990), *Azorhizobium sesbaniae* (Hilgert *et al.*, 1987) and *Bradyrhizobium japonicum* (O'Gara *et al.*, 1984) have been reported but have not been characterized. This is the first report in which the two subunits of GOGAT have been cloned and sequenced and the corresponding mutant characterized genetically and phenotypically. Although there is some phenotypic similarity with other GOGAT mutants of *Rhizobium*, the *R. etli* GOGAT mutant characterized in this work has a phenotype not previously reported (see below).

One of the objectives of this work was to analyse the physiological role of GOGAT activity in relation to the utilization of carbon and the turnover of nitrogen. We have reported that glutamine cycling through the GS-GOGAT pathway is a necessary condition for the expenditure of energy and reductive power, a process that drives carbon utilization in *Rhizobium* and in other micro-organisms (Encarnación *et al.*, 1998; Mora, 1990). It has been demonstrated that a lack of GS activity in *Rhizobium* decreases succinate utilization and oxidation, although for those experiments it was necessary to use a double mutant lacking GSI and GSII and to inhibit the remaining GSIII activity with an inhibitor

(Encarnación *et al.*, 1998). Therefore, a direct experiment to prevent glutamine cycling was to use a *R. etli* GOGAT mutant and to characterize its growth on succinate-glutamate medium and to measure its consumption of these compounds. The results of this experiment showed that in comparison to wild-type strain CFN42, the GOGAT mutant grew less well in liquid cultures with glutamate, or other amino acids as nitrogen source, and this could be directly correlated with a lower consumption of succinate and glutamate. This effect could account for the  $Asm^-$  phenotype of the mutant strain. Similar GOGAT mutants have been previously reported as having poor, or no growth in different amino acids as a sole nitrogen source (Kondorosi *et al.*, 1977; O'Gara *et al.*, 1984; Hilgert *et al.*, 1987). The latter assays were performed on solid media, where there is a limitation of oxygen. This may explain the differences with our experimental results. We have concluded that a lack of GOGAT activity in *R. etli*, by preventing glutamine cycling, impairs nitrogen and carbon metabolism and explains the significant decrease in amino nitrogen and glutamate pools, as well as the deficient growth, with each of these amino acids as sole nitrogen source. In this context, the large amounts of ammonium excreted by the GOGAT mutant in succinate-glutamate medium can be explained by the relatively higher catabolism of glutamate versus the lower utilization of succinate as carbon source (Fig. 3).

Another objective was to explore the participation of GOGAT in ammonium assimilation during protein turnover. The fact that the GOGAT mutant, in contrast to the wild-type strain, excreted ammonium under non-growing conditions (succinate alone), concomitant with a decrease in protein content (Fig. 3c) and a loss of cell viability (see Results), showed that protein degradation liberates ammonium that can not be assimilated again. According to these results, protein turnover results in ammonium turnover, and in the *de novo* synthesis of amino acids and protein, which are necessary to sustain cell viability. We are not aware of other reports about a similar role of GOGAT in protein turnover.

An additional objective using the *R. etli* GOGAT mutant was to determine in bacteroids whether the nitrogen fixed into ammonium was assimilated or excreted to the plant. Although the capacity of bacteroids to assimilate ammonium has been assumed to be ancillary to symbiosis (Kondorosi *et al.*, 1977; Osburne & Signer, 1980; Lewis *et al.*, 1990), we have demonstrated elsewhere that the ammonium assimilation pathway is active in *R. etli* bacteroids (Mendoza *et al.*, 1995, 1998) and the results presented here indicate that it is important in carbon and nitrogen metabolism during symbiosis. In the wild-type strain the amino acid found in the highest amount was glutamate, which constituted 40 and 33 %, respectively, of the total amino-nitrogen at 25 and 32 d post-inoculation (Table 3). Other workers have demonstrated that soybean bacteroids of *B. japonicum* incorporated labelled succinate first and then mainly into glutamate, and that the increase in the rate of labelled  $CO_2$  evolution coincided with a maxi-

mal accumulation of label in glutamate (Salminen & Streeter, 1987).

When the ammonium assimilation pathway is blocked, as in the GOGAT mutant, more fixed ammonium may be excreted to the plant cell, transported to the leaves and stored in the seeds (Table 4). The decrease in the amino-nitrogen pool in the GOGAT mutant suggests that part of the fixed nitrogen is assimilated by *R. etli* via the GS-GOGAT pathway, and the decreased assimilation in the GOGAT mutant could be related to the increase in nitrogenase activity observed (Table 4). Of interest is the finding that in comparison with free-living cells, GOGAT activity in *R. etli* is two- to fourfold lower during symbiosis (Table 3), which favours more of the fixed nitrogen being supplied to the plant (see Results and Table 4). Therefore, we have shown that the increase in  $\alpha$ -amino-nitrogen in bacteroids correlates with a decrease in nitrogenase activity (Mendoza *et al.*, 1995). Knight & Langston-Unkefer (1988) have found that the glutamate and glutamine content of nodules affects nitrogenase activity. The nitrogen assimilated into amino acids in the bacteroids may contribute to this effect. These and previous results (Streeter, 1987; Mendoza *et al.*, 1995, 1998) strongly challenge the view of an inactive ammonium assimilation pathway during symbiosis by demonstrating that the GS-GOGAT pathway plays a central role in the distribution of fixed nitrogen between the bacteroid and the plant.

A symbiotic phenotype was reported in a chemically induced *B. japonicum* mutant lacking GOGAT activity. The mutant did not fix  $N_2$  in symbiosis although it did in free life (O'Gara *et al.*, 1984). Since this mutant was not further characterized, it is difficult to compare these results with the data presented here.

Recently, it has been proposed that the amino acid alanine is the metabolite that transfers fixed nitrogen from soybean bacteroids to the plant (Waters *et al.*, 1998). In our experiments with *R. etli*, alanine is present in low amounts together with other amino acids in bean bacteroids (Table 3). However, during the first 25 d post-inoculation, the GOGAT mutant strain had a lower content of amino-nitrogen and, even in these conditions, alanine is not a major contributor (Table 3 and data not shown). Yet, it can not be ruled out that this amino acid is found in a low proportion because it is actively secreted, unlike glutamate. Also, in the bacteroids of this mutant, some nitrogen is still present in amino acids, indicating a secondary source of amino nitrogen not related to GOGAT activity.

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