CHARACTERISTICS OF GLUTAMINE SYNTHETASE I AND II MUTANTS OF *RHIZOBIUM*

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Mutants of *Rhizobium meliloti* 41 and AK631 deficient in either glutamine synthetase I or II were characterized for growth and acetylene reduction activity. Comparison of the various mutants indicates that either GSI or GSII is competent to provide glutamine for cellular requirements with ammonia as the nitrogen source. The acetylene reduction activity of nodules induced by some GSI⁻ or GSII⁻ mutants was equal to the wild-type activity indicating that neither GS is required for symbiotic effectiveness and that either form is sufficient for effectiveness. No effect of purines on growth was found in the GS mutants of this strain.

Characteristic isoenzymes of glutamine synthetase (GS) are found in the *Rhizobiaceae* which includes *Agrobacterium*, *Rhizobium* and *Bradyrhizobium* (3–7, 12, 13). GSI has many properties similar to the GS found in enteric bacteria (3) while GSII appears solely in the *Rhizobiaceae* (7) and has properties distinctly different from GSI (3, 4).

LUDWIG (12, 13), MORETT et al. (14), and de BRUIJN et al. (1) have isolated strains of *Rhizobium* and *Bradyrhizobium* which were GSI⁺ GSII⁻ illustrating that GSI alone is fully competent to supply the cell with glutamine. The competency of GSII to supply the cell with glutamine in *B. japonicum* 61A76 was supported by studies on the steady state kinetics of GSI and GSII (R. A. DARROW and R. R. KNOTTS, Abstracts of the 8th North American *Rhizobium* Conference, Univ. of

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Mannitoba, Winnipeg, Canada, 1981). These studies suggested that in fully derepressed cells, non-limited for ATP, over 90% of NH_4 assimilation may be mediated by GSII. Recently the gene encoding GSI in *R. meliloti* (17) and *B. japonicum* (2) has been cloned. In *R. meliloti*, recombinants with an insertion in the GSI gene lose GSI activity, retain GSII activity, grow well on ammonia and are unaffected in nodulation and nitrogen fixation.

In this paper we will show that GSII⁻ strains of *R. meliloti* 41 and AK631 and strains having very low GSI and near normal levels of GSII, are physiologically competent, illustrating that either isoenzyme can supply glutamine in this *Rhizobium* species.

LUDWIG (13) suggested that GSII in *Bradyrhizobium* sp. 32H1 may have a specific role in purine metabolism. The evidence presented was not strong and the data we will present in this paper do not support such a role for GSII. Thus, there is currently no satisfactory explanation for the presence of two catalytically active GS isoenzymes in the *Rhizobiaceae*.

MATERIALS AND METHODS

Stock cultures of *R. meliloti* were maintained on solid media containing glutamine and were grown in liquid medium as previously described (7) with nitrogen sources as noted. Cell growth was monitored by following the turbidity with a Klett-Summerson colorimeter. Cells were grown to mid-log phase, harvested and cell-free extracts prepared by disruption in a French pressure cell (6, 7). The γ -glutamyl transferase activity was measured using the assay with three buffers previously described (5, 6) and incubating for 15 min at 30°C. The isoactivity point for the adenylylated and unadenylylated form of GSI in these studies was found to be pH 7.35. The levels of GSI and GSII were determined by their differential heat stability as described earlier (6, 7). The relative amount of adenylylated GSI was determined by the amount of inhibition of transferase activity caused by 60 mM Mg^{2+} . From previous experience with *B. japonicum*, we are aware that the relationship between the percentage of magnesium inhibition and the adenylylation state as described for *E. coli* (18) does not necessarily hold for *Rhizobium*. Therefore, no attempt was made to calculate the E_n (adenylylation state).

Nodulation studies were carried out using alfalfa seedlings (variety Vernal). Seeds were sterilized for 10 min in commercial bleach to which one drop of Tween 20 has been added. After 10 washings with sterile distilled water, the seeds were transferred to 25 × 200 mm test tubes, one-half filled with Turface. A comparison of Turface and vermiculite as substrate indicated that the plant roots have 2 to 4 times more nodules in Turface. The plants were watered with half-strength Jensen's medium (20) and allowed to germinate for 3 days. Before inoculation, seedlings were thinned leaving two per tube. After inoculation with a few drops of a liquid culture of *R. meliloti*, the plants were grown at 26°C in a growth cabinet on a 16 hr light, 8 hr dark cycle. After 18 to 28 days of growth, the tubes were capped with

serum stoppers and C₂H₂ injected to 10%. Samples (0.25 ml) were removed with a syringe and ethylene was detected by gas chromatography as described previously (8).

RESULTS AND DISCUSSION

A compilation of the strains used, their phenotype and their parental strains is presented in Table 1. All strains retained GOGAT activity. In Table 2, the levels of GSII and GSI are shown as well as the ability of the adenylation system (18) to respond to ammonia. AK598, AK598R7, GY0516, ZS170, and ZS231 were GSII⁻, and AK598C41 had low GSII, while AK598C1 had moderate levels of GSII and a very reduced level of GSI. Of GSI, GY0516, AK598C41, AK598C44, and ZS231 had very reduced levels while AK598R7 had a moderate level. The adenylation system of AK598C1, which had a moderate level of GSII, appeared to be fully functional in its response to added ammonia, but none of the other mutants responded significantly. It is not known whether this is due to an impairment in the adenylation system or to some regulatory mechanism which prevents adenylation when only low levels of GS are present. The degree of adenylation after prolonged growth in glutamine was similar to that after the ammonium shock.

The effect of adenine and guanine on the rate of growth of some of these strains in various nitrogen sources is shown in Table 3. As can be seen, adenine and guanine had little or no effect on the rate of growth of any of the strains used. Thus, these results do not support the role for GSII in purine biosynthesis previously proposed by LUDWIG (13). A conclusion similar to ours was reached by SHAH et al. (16). Also, if GSII were involved in the synthesis of purines, one might expect some repression of enzyme synthesis by the addition of purine. However, the addition of adenine

Table 1. Strains of *R. meliloti*.

Strain	Growth phenotype	Nodulation phenotype	How selected	Reference
41	wild-type	Nod ⁺ Fix ⁺		(19)
AK631	wild-type	Nod ⁺ Fix ⁺	small colony variant of 41	(11)
A1	His ⁻ Str ^R	Nod ⁺ Fix ⁺	derivative of <i>R. meliloti</i> 41	(15)
AK598	His ⁻ Str ^R Gln ⁻	Nod ⁺ Fix ⁻	NTG mutagenesis of A1	(9)
AK598R7	His ⁻ Str ^R Gln ⁺	Nod ⁺ Fix ⁻	spontaneous revertant of AK598	this paper
AK598C1	His ⁺ Gln ⁺	Nod ⁺ Fix ⁺	recombinant of AK598 carrying R68.45	this paper
AK598C41	His ⁺ Gln ⁺	Nod ⁺ Fix ⁻	recombinant of AK598 carrying R68.45	this paper
AK598C44	His ⁺ Gln ⁺	Nod ⁺ Fix ⁺	recombinant of AK598 carrying R68.45	this paper
GY0516	Gln ⁻ or Glt ⁻	Nod ⁺ Fix ⁺	NTG mutagenesis of AK631	this paper
ZS170	Gln ⁻	Nod ⁺ Fix ⁺	NTG mutagenesis of AK631	this paper
ZS231	His ⁺ Gln ⁻	Nod ⁺ Fix ⁺	derivative of A1	this paper
ZS231C11	His ⁺ Gln ⁺	Nod ⁺ Fix ⁺	recombinant of ZS231	this paper

Str^R, streptomycin resistant; NTG, N-methyl-N'-nitro-N-nitrosoguanidine. Recombinants were constructed by mobilizing the *R. meliloti* chromosome using R68.45 (10).

Table 2. GSI and GSII in *R. meliloti* strains.^a

Strain	Ammonia shock ^b	Transferase activity			%Mg Inhibition
		GSII	GSI	GSI + Mg ²⁺	
		nmol min ⁻¹ mg protein ⁻¹			
AK631	—	1203	740	840	—14
	+	1332	890	115	87
AK598	—	0 ^c	86		
AK598R7	—	0	360	303	16
	+	0	376	255	32
AK598C1	—	640	130	126	0
	+	655	164	41	75
AK598C41	—	304	130	138	—6
	+	289	146	107	27
AK598C44	—	600	75		
GY0516	—	0	140		
ZS170	—	0	160	^d	
ZS231	—	0	156	135	13
	+	0	141	109	23
ZS231C11	—	0	600		

^a Cells were grown in synthetic medium containing mannitol as the carbon source and glutamate (0.1%) as the nitrogen source. For strains AK598 and AK598R7, 0.015% histidine was also present.

^b 10 mM NH₄Cl was added to the culture 15 min prior to harvest.

^c Differential heat sensitivity was used to determine GSI and II. Very small amounts are not easily determined using this assay. Thus, an activity less than 10 is considered zero.

^d ZS170 and a few strains not reported here were markedly stimulated by Mg²⁺ before and after ammonia shock. The stimulation was removed by treating the extracts with deoxyribonuclease. Thus, it may be related to DNA binding of GSI. We have not pursued this observation.

and guanine to a culture of any of the strains of *R. meliloti* described in this paper had no effect on the levels of GSII (when present), on the levels of GSI, or on the adenylation state of GSI (data not shown).

Based on these studies with *R. meliloti* it would appear that there is no specialized role for GSII in purine synthesis. Rather, based on the kinetic properties of the two GSs, DARROW has postulated that GSII is likely to serve as the major pathway of glutamine synthesis in the cells (R. A. DARROW and R. R. KNOTTS, Absts. 8th North American Rhizobium Conference, Winnipeg, Canada, 1981).

Growth studies with mutant strains

Growth studies using a variety of nitrogen sources and strains representing several GS phenotypes are presented in Table 4. The wild-type strain (AK631) grows well on a variety of nitrogenous compounds. The mutants having low GSI and no GSII (GY0516, ZS170, and ZS231) grow slowly on NH₄ and grow on NO₃ only after a long lag phase. A recombinant of ZS231 (ZS231C11) with an increased amount of GSI grows well on all of the nitrogen sources including NH₄⁺. Mutants

Table 3. Effect of purines on *R. meliloti* strains.^a

Strain	Nitrogen source	Adenine plus guanine (0.04 mM each)	Generation time (hr)	GS phenotype
AK631	Glt ^b	—	2.9	wild-type
		+	3.0	
	NH ₄ Cl	—	2.7	
		+	2.7	
AK598R7	NH ₄ Cl	—	3.9	GSII ⁻
		+	3.9	
AK598C1	NH ₄ Cl	—	5.6	GSII ⁺ , low GSI
		+	5.8	
AK598C41	Glt	—	6.3	low GSII and GSI
		+	6.6	
AK598C44	NH ₄ Cl	—	6.0	GSI ⁻
		+	5.9	
GY0516	NH ₄ Cl	—	8.1	GSII ⁻ , low GSI
		+	7.6	
ZS170	Glt	—	4.1	GSII ⁻ , low GSI
		+	4.0	
ZS231	NH ₄ Cl	—	6.6	low GSI
		+	6.9	

^a Cells were grown in the medium described for *R. meliloti* (7) with 25 mM 3-(N-morpholino) propanesulfonic acid buffer and 0.5% mannitol as the carbon source.

^b Glt, glutamate, 6 mM; NH₄Cl, 15 mM.

Table 4. Growth characteristics of GS mutants.^a

Strain	Nitrogen source generation time (hr)							
	Gln	Glt	His	Asp	Pro	Arg	NH ₄ ⁺	NO ₃ ⁻
AK631	3.3	3.5	3.3	3.8	4.4	6.7	3.3	4.8
GY0516	4.7	4.7	5.0	9.1	6.0	6.6	11.6	^b
ZS170	3.6	3.9	4.0	4.8	4.6	8.3	10.0	^b
ZS231	7.1	6.1	6.7	9.8	7.6	8.5	13.4	^b
ZS231C11	3.5	3.8	3.6	5.5	4.8	6.1	4.5	5.1
AK598C41	4.9	5.4	5.7	6.8	7.0	6.8	6.9	^b
AK598C1	4.3	4.4	4.5	4.9	4.9	6.6	5.9	5.3
AK598C44	6.6	11.2	6.7	9.5	13.2	13.6	7.5	11.0

^a See Table 3 for growth medium. Nitrogen sources were as follows: gln, NH₄⁺ (as ammonium sulfate) and his, 5 mM; all others, 10 mM.

^b Growth occurs after a long lag period.

with low GSI and moderate levels of GSII (AK598C41, AK598C44, and AK598C1) grow on NH₄⁺ and NO₃⁻ although not as well as the wild type strain. AK598C1 and AK598C44 have GSI levels equal to or only slightly greater than their Gln⁻ parent,

Table 5. Acetylene reduction activity of nodulated plants.^a

Strain	C ₂ H ₂ Reduction nmol hr ⁻¹ plant ⁻¹		Nodules/plant
	18 days	28 days	
AK631	950	1530	45 ± 20
GY0516	690	1500	57 ± 33
ZS170	870	1450	59 ± 26
ZS231	490	440	95 ± 42
ZS231C11	120	750	88 ± 26
AK598C1	770	660	103 ± 45
AK598C41	—	—	59 ± 28
AK598C44	800	1410	101 ± 46

^a The results of the nodulation studies are the average of at least three separate experiments with at least six plants per experiment. That the inoculated strain could be recovered from nodules was demonstrated with strains GY0516, ZS170, AK598C1, and AK598C44. A nodule was selected at random from six separate plants. They were surface sterilized, crushed and streaked on agar plates. A colony was selected and cultured and the GS was determined. All colonies retained the original phenotype.

AK598. Thus, both GSI and GSII appear competent to supply the cell with glutamine.

Nodulation studies

The effectiveness of nodules as determined by C₂H₂ reduction with intact plants is shown in Table 5. These results show that strains with only low amounts of GSI are fully effective (GY0516 and ZS170), and that a strain with predominantly GSII (AK598C44) was also fully effective. This latter recombinant strain has GSI activity no higher than the mutant parent (AK598). Although not recorded herein, the biosynthetic activity of the small amount of GSI of AK598C44 was found to be less than 20% of the biosynthetic activity expected for an equivalent amount of GSI activity in the wild type based on the transferase assay. Thus, the actual synthetic capacity of GSI from this strain is negligible. These results suggest that the catalytic activity of either GSI or GSII is sufficient to provide the bacterial cell with enough glutamine for the transition from free living to symbiotic function and that neither GSI nor GSII specifically, is required to sustain an effective symbiosis.

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