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The Enhancement of Ammonium Assimilation in *Rhizobium etli* Prevents Nodulation of *Phaseolus vulgaris*

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The modification of the ammonium assimilation pathway of *Rhizobium etli* (GS-GOGAT) by adding an additional ammonium assimilation enzyme, GDH, strongly affects its symbiotic interaction with beans. The plasmid pAM1a, based in the stable vector pTR101 (M. Weinstein, R. C. Roberts, and D. R. Helsinki, J. Bacteriol. 174,7486-7489, 1992), containing the *Escherichia coli gdhA* gene flanked by two transcription-translation terminators was constructed. The expression of GDH in both, the wild type (CFN42/pAM1a) and a *ntrC*$^-$ mutant (CFN2012/pAM1a) *R. etli* strains, gave a similar metabolic effect, i.e., high GDH and reduced GOGAT activities, and an increased synthesis and excretion of several amino acids. The total inhibition of bean nodulation was observed when the minimum optimal inoculum of *R. etli* CFN42/pAM1a was used; however, an effective symbiosis occurred with the CFN2012/pAM1a mutant strain. While a total inhibition of the induction of the *nodA* gene by bean root exudate or by naringenin was observed in the CFN42/pAM1a strain, at 10 mM ammonium, the CFN2012/pAM1a showed an optimal *nodA* gene induction. A correlation between *nodA* gene induction, Nod factor production, and nodulation was observed. We conclude that in *R. etli*, there is a down-regulation of *nod* gene expression and nodulation when a high internal nitrogen content is built up by the presence of a functional GDH and that NtrC is involved in such regulation. An instability of the plasmid harboring the *gdhA* gene was observed during symbiosis, indicating a strong selection against cells containing this plasmid.

Additional keywords: glutamate dehydrogenase, nitrogen regulation of *nod* genes, *Rhizobium*–legume symbiosis, *Rhizobium* nitrogen metabolism.

In contrast to microorganisms that utilize the glutamate dehydrogenase (GDH)-glutamine synthetase (GS) ammonium assimilation pathway when growing in ammonium excess, virtually all microorganisms 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, since GS has a lower Km for this substrate than does GDH. Use of this pathway may benefit the organism because ammonium derived from nitrogen fixation is usually present in limited amounts. In common with the free-living diazotrophs, many *Rhizobium* species assimilate ammonium through the GS-GOGAT pathway (Kondorosi et al. 1977; Ludwig 1978; Osburne and Signer 1980; O'Gara et al. 1984), and all of the glutamate auxotrophs that have been isolated from these species are affected in their GOGAT activity (Kondorosi et al. 1977; Ludwig 1978l; Osburne and Signer 1980; Donald and Ludwig 1984; O'Gara et al. 1984). We have reported that in *Rhizobium leguminosarum* bv. *phaseoli*, ammonium is assimilated through the GS-GOGAT pathway and no GDH activity is present (Bravo and Mora 1988). Recently, type I strains from this biovar, which nodulate beans have been taxonomically reclassified as *R. etli* (Segovia et al. 1993), and in this paper we will use this current nomenclature.

It is generally accepted that ammonium assimilation is not functional in *Rhizobium* when the bacteria are engaged in symbiosis with legumes. Biochemical and genetic evidence has demonstrated that one of the GS isozymes, GSII, is not functional in symbiotically associated *Rhizobia*. The activity of GSII was not detected in *R. etli* isolated from bean nodules (Moreno et al. 1991), and in *R. meliloti* obtained from alfalfa nodules. Neither the transcription of *glnII*, measured by using a *glnII–lacZ* gene fusion (de Bruijn et al. 1989) nor the GSII protein, determined with anti-GSII-specific antiserum (Shatters et al. 1989), were present. However, the GOGAT activity has been detected in bacteroids from nodules formed on different legume species (Brown and Dilworth 1975). Ammonium assimilation mutants of *R. meliloti*, isolated after UV mutagenesis, which lack GOGAT activity were not altered in the symbiosis with alfalfa (Osburne and Signer 1980). Nevertheless some revertant strains obtained from those *R. meliloti* mutants, which increased their GDH activity, were defective in nodulation (Osburne and Signer 1980) but no explanation was given for such defect. In addition, GSI activity is present in *R. etli* bacteroids isolated from bean nodules (Moreno et al. 1991). The abolition of GSI and/or GSII activities by genetic manipulation has different effects on nodulation and nitrogen fixation, depending on the specific symbiosis in question. For instance, in *R. meliloti*, the lack of GSI and GSII affects neither nodulation nor nitrogen fixation; a third GS isozyme, GSIII, has been found in that

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microorganism (de Bruijn et al. 1989). In alfalfa root nodules formed by a *R. meliloti glnAglnII* double mutant the GSIII protein was detected, using anti-GSIII-specific antiserum, (Shatters et al. 1993). GSIII has also been found in *R. etli* (Espín et al. 1990), but its role in symbiosis is unknown. In *Bradyrhizobium japonicum*, a mutant lacking both GSI and GSII is severely impaired in both nodulation and nitrogen fixation (Carlson et al. 1987); GSIII is absent in this species.

We believe that the general acceptance for a lack of ammonium assimilation by *Rhizobium* during symbiosis has prevented more detailed studies on the active nitrogen metabolism of the bacteria in planta. There is ample evidence indicating that bacteroids synthesize different nitrogen metabolites during symbiosis (Salminen and Streeter 1987). For example, glutamate and aspartate stimulate both nitrogen fixation and respiration in soybean bacteroids following their transamination to aspartate and the subsequent deamination of this amino acid by aspartase (Kouchi et al. 1991). It has also been shown that the 4-aminobutyrate pathway, the GABA shunt, is partly responsible for the catabolism of glutamate in *B. japonicum* bacteroids (Kouchi et al. 1991). Studies on the exchange of metabolites across the peribacteroid membrane in pea root nodules suggest the participation of a transaminase activity associated with this membrane (Rosendahl et al. 1992), in addition to the glutamate oxaloacetate transaminase that participates in the malate/aspartate shuttle between plant and bacteroid (Appels and Haaker 1991). A *R. meliloti* mutant which lacks aspartate amino transferase is impaired in nitrogen fixation (Rastogi et al. 1991). The synthesis of other nitrogen metabolites, such as rhizopines (Murphy et al. 1988), has been reported to occur in the bacteria during symbiosis. Our own studies, in other microorganisms, have shown that ammonium assimilation is essential for the utilization and metabolism of carbon (Mora 1990).

We have reported the effect in symbiosis of the introduction of the *gdhA* gene from *Escherichia coli* (Bravo et al. 1988). In distinction to what was reported for *R. meliloti* revertant mutant strains with increased GDH activity (Osburne and Singer 1980), in the *R. etli* wild-type strain expressing GDH activity we reported the formation of different type of nodules depending on the presence or absence of GDH activity (Bravo et al. 1988). Most of the nodules formed were effective but contained bacteria that had lost the plasmid, and also small white nodules with bacteroids that expressed GDH activity, severely impaired on nitrogen fixation, were formed (Bravo et al. 1988). However, since the plasmid used in that work (Bravo et al. 1988) was unstable, it was difficult to draw definitive conclusions.

In this work we show that *R. etli* bearing a stable plasmid containing only the *gdhA* gene, expresses a functional GDH in minimal media and represses nodulation when engaged in symbiosis. We found that *ntrC*, a member of the general nitrogen regulatory system, reverts the GDH effect and we propose that *ntrC* is involved in the nitrogen regulation of nodulation in *R. etli*.

RESULTS

A stable plasmid for *Rhizobium* bearing the *E. coli gdhA* gene.

The plasmid pTR101 (Table 1) is a mini-RK2 vector which carries the *par* stabilization locus, which allows it to be stably maintained in *R. meliloti* both in and ex planta (Weinstein et al. 1992), as well as in other bacteria (Roberts et al. 1990; Davis et al. 1992) when grown in the absence of Tc selection.

We determined >99% stability of this vector pTR101 in *R.*

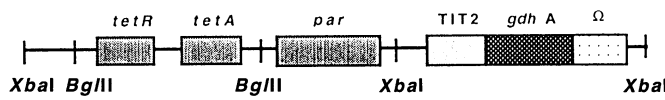

Fig. 1. Diagrammatic representation of pAM1a plasmid. The diagram is not drawn to scale. The estimated size of the plasmid is 13.2 kilobases. Shaded boxes represent relevant genes of the plasmids pTR101 (Weinstein et al. 1992), which was used as the vector for constructing the pAM1a: *tetR tetA*, tetracycline resistance; *par*, 0.8-kb RK2 stabilization locus. A *XbaI–XbaI* 4.8-kb fragment containing the *gdhA Escherichia coli* gene (1.5 kb) flanked by the transcription-translation terminators: T1T2 and Ω (Sm^r/Sp^r) was cloned into the *XbaI* site of pTR101, thus generating pAM1a.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
R. etli		
CFN42	Wild-type strain; Sm^rNal^r	Bravo and Mora 1988
CFN2012	CFN42 derivative, *ntrC* ::Tn5	Moreno et al. 1992
UBP102	CFN42 derivative with *nod A* ::MudII PR13 in p42d(pSym)	Vázquez et al. 1991
DEM301	CFN2012 derivative with *nodA*::MudIIPR13 in p42d (pSym)	This work
E. coli		
HB101	F^-*hsd S20-recA13*	Boyer and Roulland-Dussoix 1969
Plasmids		
pRK2013	Col E1 *mob*$^+$$Tra^+$(RK2); Km^r	Figurski and Helinski 1979
pSAE4	pBR322 with a 3.6-kb *Hin*dIII-*Pst*I fragment containing *E. coli gdhA* gene	Sánchez-Pescador et al. 1982
pMW157	pIC20H with the T1T2 transcription-translation terminator cloned in the *Sma*I site and the Ω (Sm^r/Sp^r) fragment cloned in the *Eco*RI site	Williams et al. 1988
pAM300	pMW157 with 1.5-kb *Dde*I-*Dde*I fragment from pSAE4 containing the *E. coli gdhA* gene, cloned in the *Kpn*I site	This work
pTR101	pTR100 mini-RK2 vector with the 0.8 kb stability locus; Tc^r	Weinstein et al. 1992
pAM1a	pTR101 with 4.8-kb *Xba*I-*Xba*I fragment from pAM300 containing the *E. coli gdhA* gene flanked by transcription-translation terminators, cloned in the *Xba*I site; Tc^rSp^r	This work
pRP30	Cosmid pSM991.44 with *nodA* ::MudII PR13; Tc^r Cm^r	Vázquez et al. 1991

etli CFN42 when grown on PY medium without selection for over 16 generations, as well as in the nodules of *Phaseolus vulgaris* inoculated with CFN42/pTR101. The size and color of the nodules formed by CFN42/pTR101 strain are similar to those formed by the wild-type strain, without a plasmid.

The *E. coli gdhA* gene (Valle et al. 1984) was cloned into the vector pTR101 (Table 1) flanked by two transcription-terminators that allowed the expression of GDH in *Rhizobium*, derived only from its own promoter (plasmid pAM1a) (Fig. 1). The pAM1a plasmid is stable (>99%) in the *R. etli* strains tested grown on PY medium or MM without selection.

Phenotypic characteristics of *R. etli* strains expressing the *E. coli gdhA* gene.

When the *R. etli* strains with or without the pTR101 vector were grown on minimal media (MM) with different nitrogen sources they showed similar growth rate, specific activities of GDH and GOGAT and amino acid content.

We compared the phenotype of the control strain CFN42/pTR101 with that of the strain carrying the *gdhA* gene (CFN42/pAM1a). In each culture, with a different nitrogen source, the CFN42/pAM1a strain gave a lower growth rate than the control strain (Table 2). The GDH and GOGAT specific activities were determined in both strains after 10 (exponential phase) and 24 h (stationary phase) of growth in MM with different nitrogen sources. The CFN42/pAM1a strain expressed a high GDH activity in ammonium and it was partially repressed (50%) at 10 h of growth in glutamate (Table 2). The GOGAT activity was more drastically affected in the CFN42/pAM1a strain, it was only 17 and 13% when grown in ammonium and glutamate for 10 or 24 h, respectively, as compared to that of the CFN42/pTR101 control strain (Table 2).

The wild-type strain expressing GDH (CFN42/pAM1a) accumulates higher intracellular glutamate content at 24 h of growth in ammonium (Table 3). The total concentration of other amino acids was also increased in the CFN42/pAM1a strain as compared to the control strain (CFN42/pTR101) (Table 3). Furthermore, glutamate and other amino acids were excreted to the medium by the CFN42/pAM1a strain (Table 3). The higher glutamate pools present could be involved in down regulating the GOGAT activity in the CFN42/pAM1a strain (Tables 2 and 3).

The effect of *ntrC*, a member of the general nitrogen regulatory system, on GDH expression was evaluated. The GDH expression in an *R. etli* mutant strain lacking NtrC (CFN2012) (Moreno et al. 1992) was determined, both at 10 (exponential phase) and 24 h (stationary phase), of growth in ammonium. The CFN2012/pAM1a strain gave a slightly higher growth rate than the control strain (CFN2012/pTR101) and it expressed a high GDH activity similar to that expressed in the CFN42/pAMla strain (Table 2). The GOGAT activity was also repressed in the *ntrC*$^-$ mutant strain expressing GDH, it was 18 to 25% as compared to that of the CFN2012/pTR101 control strain (Table 2). The *ntrC*$^-$ mutant strain expressing GDH (CFN2012/pAM1a) accumulated similar amounts of glutamate and other amino acids, than the wild-type strain expressing GDH (CFN42/pAM1a) (Table 3). Also, the CFN2012/pAM1a strain excreted a higher amount of glutamate and other amino acids to the medium than the control strain (Table 3); and this is similar to what was shown for the CFN42/pAM1a strain (Table 3). Therefore, the expression of the *E. coli gdhA* gene in an *R. etli ntrC*$^-$ mutant background had a similar metabolic effect than in the wild-type background.

Symbiotic phenotype of *R. etli* strains expressing the *E. coli gdhA* gene.

In this work we used the CFN42/pAM1a strain as inoculum, first in the usual high concentration (10^9 cells/plant) and the nodulation was diminished and delayed. Most of the nodules formed were similar in size and color to those formed by the wild-type strain, and 100% of the bacteria isolated from these nodules were Tc^S; very few small, white nodules, containing a mixed population of bacteria most of which had lost the pAM1a plasmid, were found. Microscopically, the abnormal undeveloped nodules showed a reduced number of infected cells with smaller symbiosomes and there were fewer bacteroids in each symbiosome. Although the pTR101 vector was 100% stable in the nodules, there was a strong selection against it when this plasmid carried the *gdhA* gene during the symbiosis. Similar observations were reported before (Bravo *et al* 1988).

Table 2. Phenotype of *Rhizobium etli* expressing the *Escherichia coli gdhA* gene

Strain	Nitrogen source[a]	Doubling time (h)	Time (h)	Specific activity (nmol NADP/ min/mg protein) GOGAT	GDH
CFN42/pTR101	Ammonium	4.0	10	65	0
			24	64	0
CFN42/pAM1a		6.0	10	11	248
			24	10	275
CFN42/pTR101	Glutamate	3.5	10	30	0
			24	23	0
CFN42/pAM1a		4.0	10	4	99
			24	6	160
CFN2012/pTR101	Ammonium	4.0	10	44	0
			24	49	0
CFN2012/pAM1a		3.5	10	11	195
			24	9	146

[a] Minimal media batch liquid cultures of *R. etli* were grown at 30°C, shaken at 200 rpm with the indicated nitrogen source at 10 mM concentration.

Table 3. Amino acid concentration of *Rhizobium etli* expressing the *Escherichia coli gdhA* gene

Strain	Intracellular[a] (µmol/mg protein) Glutamate	Other amino acids[b]	Extracellular[a] (µmol/mg protein) Glutamate	Other amino acids[b]
CFN42/pTR101	0.007	0.004	0.009	0.010
CFN42/pAM1a	0.035	0.010	1.025	0.076
CFN2012/pTR101	0.010	0.002	0.019	0.017
CFN2012/pAM1a	0.063	0.016	1.124	0.123

[a] Minimal media batch liquid cultures with 10 mM ammonium as nitrogen source were grown for 24 h at 30°C shaken at 200 rpm, and the amino acids content was determined both intracellularly and in the culture medium.

[b] The sum of the concentration of other atino acids such as aspartate, treonine, serine, glutamine, glycine and alanine. The amino acid concentration was determined in a least three replicates for each condition, representative results are shown.

Based in those results, we analyzed the symbiotic phenotype of the CFN42/pAM1a, as compared to the CFN42 wild-type strain, and the CFN42/pTR101 control strain, using as inoculum the minimum concentration (10^7 cells/plant) of the bacterial suspension required for optimal nodulation by the wild-type strain. The rationale for this experiment was that if the plasmid harboring GDH is lost after inoculation, such a condition would permit a drastic reduction in the fraction of the bacterial population in the inoculum which might have lost the pAM1a plasmid. The parameters that were analyzed were: nodulation, nitrogen fixation, and plant yield, and they are shown in Table 4. The values obtained for the CFN42 wild-type strain were similar to those from the control strain bearing the pTR101 vector (Table 4). When the CFN42/pTR101 strain was used as inoculum it occupied 100% of the nodules formed, indicating the stability of this plasmid during symbiosis. When the CFN42/pAM1a was used as inoculum, the nodulation of *P. vulgaris* was totally inhibited (Table 4). The bean plants inoculated with this strain were chlorotic and their dry weight was lower relative to the plants inoculated with the CFN42/pTR101 strain, and similar to the uninoculated control plants, both at 18 and 25 days postinoculation (Table 4). At a later stage of the symbiosis, 30 days postinoculation, the plant yield of the control strain is twofold higher (0.730 g) relative to the yield (0.350 g) of the plants inoculated with the CFN42/pAM1a strain, which are similar to the parameters of uninoculated plants.

The *R. etli ntrC*$^-$ mutant strain (CFN2012) is not affected in its symbiotic process with *P. vulgaris*; it has a normal symbiotic phenotype (Moreno et al. 1992). In this work we analyzed the symbiotic phenotype of the CFN2012/pAM1a, as compared to the CFN2012 and the CFN42/pTR101 control strains, using the minimum optimal inoculum concentration (10^7 cells/plant). The nodulation, nitrogen fixation, and plant yield values obtained for the CFN2012 mutant strain were similar to those from the strain bearing the pTR101 vector (Table 4). In addition, these values were very similar to those obtained in the wild-type control strains (Table 4), thus proving that the lack of a functional NtrC protein does not affect the *R. etli–P. vulgaris* symbiosis (Moreno et al. 1992), even with the minimum inoculum concentration. In distinction to the wild-type strain bearing the pAM1a plasmid, when the CFN2012/pAM1a mutant strain was used as inoculum the symbiotic phenotype was not affected, the values obtained were similar to those from the control strains (Table 4). When either the control strain CFN2012/pTR101 or the CFN2012/pAM1a strain were used as inoculum they occupied 100% of the nodules formed, indicating the stability of these plasmids during symbiosis when there is no selection against it.

Nod gene induction and Nod factor production in *R. etli* strains expressing the *E. coli gdhA* gene.

It has been reported that the flavonoid induction of common *nod* genes is repressed by high concentrations of ammonium both in *R. meliloti* (Dusha et al. 1989; Dusha and Kondorosi 1993) and *B. japonicum* (Wang and Stacey 1990). In *R. meliloti*, the regulatory circuit for this *nod* gene regulation by nitrogen involves the product of *ntrC* which exerts its effect via *syrM–nodD3* regulatory genes (Dusha and Kondorosi 1993). Since the nodulation of *P. vulgaris* by the *R. etli* wild-type strain harboring the GDH gene was inhibited and this effect was reverted by the *ntrC*$^-$ mutation, we investigated the possibility that this inhibition might be explained by a decrease in *R. etli nod* gene induction, possibly mediated by NtrC, and with the corresponding decrease in the production of Nod factors which are synthesized by the products of the structural *nod* genes and are essential during the initial steps of the nodulation process (Brewin 1991).

The pTR101 and the pAM1a plasmids were separately conjugated into the UBP102 strain (Vázquez et al. 1991), which is a derivative of the *R. etli* wild-type strain, or into the DEM301 strain, which is a derivative of the *R. etli ntrC*$^-$ mutant strain, both containing *nodA–lacZ* gene fusion in the symbiotic plasmid (Table 1). The β-galactosidase activity expressed in the strains bearing the *gdhA* gene, after they were incubated for 6 h in MM with different ammonium concentrations and containing either bean root exudate or naringenin, a flavonoid *nod* gene inducer in *R. etli* exuded by *P. vulgaris* roots (Hungria et al. 1992), were compared to that found in the control strains bearing the pTR101 vector (Fig. 2). The latter were used as controls since they had levels of β-galactosidase induction similar to those of the UBP102 or DEM301 strains (without a plasmid) under all the conditions

Table 4. Symbiotic phenotype in the *Rhizobium etli–Phaseolus vulgaris* symbiosis

Strain	dpi[a]	Nodulation[b] (g)	Nitrogenase specific activity (μmol ethylene/h/g nodule)	Yield[c](g)
CFN42	18	0.020 (±0.005)	80 (±10)	0.234 (±0.048)
	25	0.040 (±0.005)	70 (±8)	0.442 (±0.082)
CFN42/pTR101	18	0.027 (±0.004)	65 (±8)	0.234 (±0.043)
	25	0.046 (±0.011)	53 (±5)	0.500 (±0.157)
CFN42/pAM1a	18	0	0	0.198 (±0.033)
	25	0	0	0.364 (±0.056)
CFN2012	18	0.024 (±0.004)	76 (±12)	0.250 (±0.046)
	25	0.043 (±0.008)	55 (±10)	0.450 (±0.096)
CFN2012/pTR101	18	0.022 (±0.005)	49 (±12)	0.297 (±0.051)
	25	0.042 (±0.011)	51 (±9)	0.545 (±0.065)
CFN2012/pAM1a	18	0.024 (±0.004)	61 (±12)	0.294 (±0.067)
	25	0.045 (±0.010)	54 (±8)	0.529 (±0.082)
Uninoculated	18	0	0	0.230 (±0.058)
	25	0	0	0.345 (±0.032)

[a] Days postinoculation.
[b] Nodule dry weight (±S.D.)
[c] Plant dry weight (±S.D.)

tested. In the different media used for this experiment, containing from 0.2 to 30 mM ammonium as the nitrogen source, the bacterial strains were in the exponential growth phase after 6 h, and their growth rate was not influenced by the presence of root exudate or naringenin. The GDH was similarly expressed in the UBP102/pAM1a and DEM301/pAM1a strains after 6 h of growth in each of the media tested, it was not influenced by the ammonium concentration in the media and the values were similar to those shown in Table 2. The basal β-galactosidase activity present in each strain in control conditions, in the absence of any inducer, was similar in every strain.

In addition the Nod-factor production in the *R. etli* strains (wild type or *ntrC*⁻) bearing the *gdhA* gene was determined (Poupot et al. 1995), after they were incubated for 6 h in MM with 10 mM ammonium and naringenin, and it was compared to the Nod factor production in the control strains bearing the pTR101 vector (Fig. 3).

The *nodA* gene induction was slightly repressed by high ammonium concentrations in the control strain UBP102/pTR101; there was 50 to 65% induction of β-galactosidase activity at the highest ammonium concentration tested (30 mM), when compared to the activity present in lower ammonium concentration, with naringenin or root exudate (Fig. 2A). The *nodA* gene induction was more drastically repressed in the strain expressing GDH (UBP102/pAM1a), it showed only 30 to 40% induction at 7.5 mM ammonium and a total repression (0%) at 10 mM ammonium (Fig. 2A). In addition, the specific activity of β-galactosidase expressed in the UBP102/pAM1a strain, when incubated with exudates or naringenin, was lower than in the control strain (UBP102/pTR101), even in the lower ammonium concentrations (0.2 to 5 mM) (data not shown).

The Nod factors were clearly produced in the control wild-type strain (CFN42/pTR101) when incubated with naringenin in MM plus 10 mM ammonium (Fig. 3), in this condition there was a considerable induction (65%) of *nod* gene expression (Fig 2A). In the same condition, where the wild-type strain expressing GDH (CFN42/pAM1a) totally repressed the *nod* gene induction (Fig. 2A) the Nod factors were not produced (Fig. 3).

The *ntrC*⁻ control mutant strain (DEM301/pTR101) showed a similar very high induction of the *nodA* gene than the wild-type control strain, it was only diminished to 70% when incubated in the highest ammonium concentration tested (30 mM) (Fig. 2B). The *ntrC*⁻ mutant strain expressing GDH (DEM301/pAM1a) showed a very high induction of β-galactosidase in every ammonium concentration tested (Fig. 2B). The specific β-galactosidase activity expressed in the DEM301/pAM1a strain when incubated with exudates or with naringenin was the highest expressed in every condition tested as compared to that expressed in the other strains (data not shown). The *ntrC*⁻ mutant strains, either expressing GDH (CFN2012/pAM1a) or not expressing it (CFN2012/pTR101), produced similar amount of Nod factors after incubation with naringenin in MM plus 10 mM ammonium (Fig. 3), where they showed maximal induction of *nod* genes (Fig. 2B).

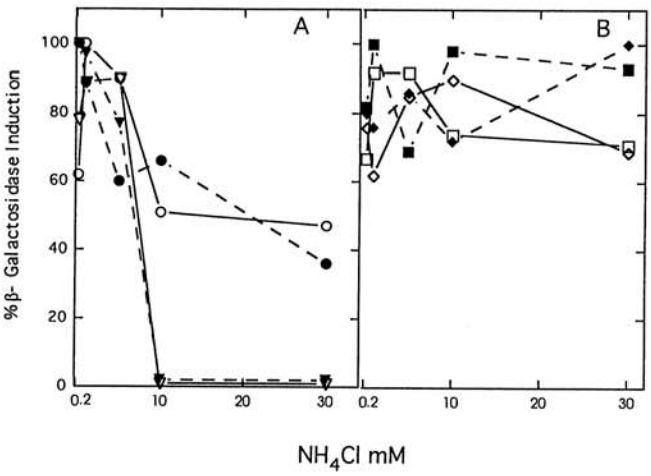

Fig. 2. Effect of ammonium concentration on the induction of *nodA*-gene from *Rhizobium etli* strains expressing GDH. The β-galsactosidase activity present in the wild-type strain (UBP102) **(A)** or in the *ntrC*⁻ mutant strain (DEM301) **(B),** containing a *nodA–lacZ* gene fusion each, and bearing either the vector pTR101 (control strains) or the plasmid pAM1a, with the cloned *gdhA* gene, was measured after 6 h of incubation in MM with different ammonium concentrations, starting with 0.2 mM. The enzyme activity was measured in at least four replicates for each condition. The percentage of β-galactosidase induction was calculated after subtracting the basal β-galactosidase activity present in the control cultures (without inducer) of each condition and considering as 100% the highest β-galactosidase activity obtained for each strain in the presence of either root exudate or naringenin. A, (open circle, closed circle) UBP102/pTR101, (inverted open triangle, inverted closed triangle) UBP102/pAM1a. B, (open square, closed square) DEM301/pTR101, and (open diamond, closed diamond) DEM301/pAM1a. Solid lines and white symbols: bean root exudate. Dotted lines and black symbols: 1.2 μM naringenin.

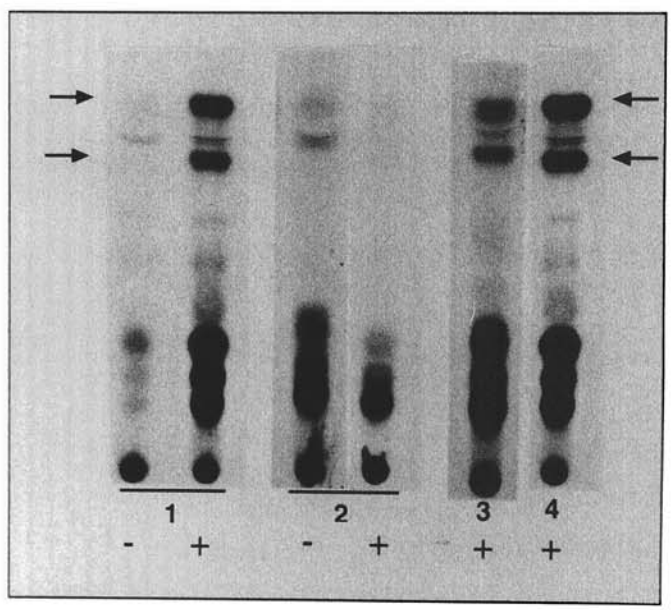

Fig. 3. Nod factors production in *Rhizobium etli* strains expressing GDH. Autoradiography of the Thin-layer chromatography performed to dtermine the Nod factors present in the supernatant of different *R. etli* cultures grown for 6 h in MM with 10 mM ammonium plus C^{14}-glucosamine, either induced with naringenin or uninduced. (1) CFN42/pTR101, (2) CFN42/pAM1a, (3) CFN2012/pTR101, (4) CFN2012/pAM1a. (–) without inducer, (+) with 1.2 μM naringenin. The uninduced cultures of the CFN2012/pTR101 and CFN2012/pAM1a showed a similar pattern as that observed for the other strains in lanes 1 (–) and 2 (–). Arrows indicate the migration of *R. etli* Nod factors.

The bean root exudate isolated after inoculation with either the CFN42/pTR101 control strain or the CFN42/pAM1a strain, gave the same induction of β-galactosidase activity from the UBP102 strain (data not shown). Therefore, the possible excretion of amino acids of the CFN42/pAM1a strain to the rhizosphere did not affect the exudation of *nod* gene inducers by the plant.

DISCUSSION

As stated previously, the problem of ammonium assimilation and of the general status of nitrogen metabolism in bacteroids has not been solved. It is important to determine if ammonium is assimilated not only by the plant, but also by the bacteroids and, furthermore, what would be the result of the competition for the expenditure of energy in nitrogen fixation over ammonium assimilation. In this context, the aim of this work was to metabolically engineer *R. etli* by adding a second ammonium assimilation step, i.e., the GDH, and to determine its effect in the symbiotic process.

The involvement of *ntrC*, a member of the general nitrogen regulatory system, as part of the regulatory circuit operating in a *R. etli* strain with an increased ammonium assimilation, was evaluated. Therefore, the expression of the *E. coli gdhA* gene from its own promoter in *R. etli,* wild type or *ntrC*$^-$ mutant strain, grown on MM plus ammonium was determined (Table 2). The *ntrC*$^-$ mutation gave no effect in the growth rate (Table 2). The GDH activity was highly expressed in both strains bearing the pAM1a plasmid (Table 2). GOGAT activity was affected similarly, sixfold decrease, in both strains (Table 2). There was a similar intracellular accumulation, as well as an excretion, of glutamate and other amino acids in the strains expressing GDH (Table 3). The specific activity of GDH is 20-fold higher as compared to GOGAT (Table 2), therefore GDH may contribute significantly to an increased glutamate synthesis (Table 3) in both the wild type and *ntrC*$^-$ mutant strains.

A total inhibition of bean nodulation was observed when the minimum amount of wild-type bacteria required for optimal nodulation and bearing a more stable plasmid carrying the *E. coli gdhA* gene (CFN42/pAM1 strain), was used as inoculum (Table 4). Nevertheless, when the *ntrC*$^-$ mutant strain bearing the plasmid with GDH was used as inoculum a normal nodulation and nitrogen fixation occurred (Table 4).

These data lead us to propose that the capacity to assimilate ammonium by *Rhizobium* has to be precisely regulated for infection and/or nodulation to occur. It appears that an ineffective symbiosis results, not only when a nitrogen compound is added to the rhizosphere, but, as this paper shows, also when an increased amount of ammonium can be assimilated by the bacteria prior to symbiosis. Then the bacteria must be nitrogen deprived to be able to nodulate. Since there was a similar metabolic effect in the wild-type strain and in the *ntrC*$^-$ mutant strain of *R. etli* expressing GDH (Tables 2 and 3), the reversion to a normal symbiosis, when expressing GDH in an *ntrC*$^-$ mutant background, was interpreted in regard to the effect of NtrC as regulator of key symbiotic genes, such as the *nod* genes.

The inhibition of nodulation by the *R. etli* wild-type strain expressing the *gdhA* gene may be explained by the greatly reduced inducibility of *nod* genes, which are regulated by the high nitrogen content (Fig. 2A). This reduced induction inhibits the synthesis of the Nod factors (Fig. 3), that are essential during the initial steps of the nodulation process (Brewin 1991). We consider that our data are consistent with the repressive effects of high ammonium concentrations on *nod* gene expression in other rhizobia (Dusha et al. 1989; Dusha and Kondorosi 1993; Wang and Stacey 1990). The flavonoid induction of common *nod* genes is repressed by high concentrations of ammonia (30 mM or more), both in *R. meliloti* (Dusha et al. 1989; Dusha and Kondorosi 1993) and *B. japonicum* (Wang and Stacey 1990), and this repression was shown to be mediated by the transcriptional activators *nodD3* or *nodD*, respectively. In contrast to the higher ammonium concentrations used to inhibit *nod* gene induction in *R. meliloti* (Dusha et al. 1989, Dusha and Kondorosi 1993) and *B. japonicum* (Wang and Stacey 1990), in this work we have shown that in the wild-type strain of *R. etli* expressing GDH *nodA* gene induction is totally repressed in the presence of only 10 mM ammonium (Fig. 2A) and in this condition the Nod factors are not produced (Fig. 3). Our system, which results in an increased endogenous pool of nitrogen metabolites, like several amino acids (Table 3), is a more sensitive one than when GDH is absent and higher extracellular ammonium concentrations are required to inhibit *nod* gene induction by flavones. In contrast to our results for *R. etli* and those for *R. meliloti* (Dusha et al. 1989, Dusha and Kondorosi 1993) and *B. japonicum* (Wang and Stacey 1990), it was reported that the *nodD* and *nodABC* genes of *Rhizobium leguminosarum* bv. *viciae* are not regulated by combined nitrogen (Baev *et al*, 1992). It remains to be investigated if *R. leguminosarum* bv. *viciae* with an increased capacity to assimilate ammonium, which is a more sensitive system as we have shown, represses *nod* gene induction.

We propose that in *R. etli*, the regulator NtrC is involved in the down regulation of common *nod* genes (*nodA*) (Fig. 2A), which correlates with the absence of Nod factor production (Fig. 3), that we found when GDH was expressed (Table 2) and when higher amino acid pools were present (Table 3) in the wild-type strain. A *ntrC*$^-$ mutant strain expressing GDH showed a normal symbiotic phenotype (Table 4) and Nod factor (Fig. 3) production, and a high *nodA* gene induction both at low and at high ammonium concentrations (Fig. 2B). Since NtrC is involved in the regulation of other genes of nitrogen metabolism other regulatory effects of this regulator in *R. etli*, cannot be excluded.

In the case of *R. meliloti* NtrC was shown to act as an activator of *nod* genes, via the *syrM–nodD3* genes, at low ammonium concentrations (Dusha et al. 1989, Dusha and Kondorosi, 1993). It was proposed that transcriptional regulation of *nodD3* by *SyrM* may be physiologically regulated by the availability of fixed nitrogen derived from the plant (Brewin 1991). It has yet to be precisely defined which are all the components of the nitrogen-dependent regulatory circuit of nodulation in *R. etli*.

Similar to our previous work (Bravo et al. 1988), here we found a strong selection against the plasmid bearing the *gdhA* gene, during the symbiosis. However, as this paper shows, the effect in symbiosis is more drastic when a "stable" plasmid bearing this gene was used, since a total inhibition of nodulation was observed at low inoculum concentration of the wild-type strain (CFN42/pAM1a) (Table 4). The delayed and di-

minished nodulation observed with a high inoculum of CFN42/pAM1a lead us to propose that a smaller fraction of the bacterial population which had lost the pAM1a plasmid is present. The mechanisms involved in the selection against the GDH "stable" plasmid remain to be investigated.

The different symbiotic phenotypes observed when we used either a low (Table 4), or a high inoculum concentration of the CFN42/pAM1a strain lead us to conclude that the alteration in the ammonium assimilation pathway of *R. etli* can affect at least two different processes during the symbiosis with beans. First, the initial stages of nodule formation are blocked, as a result of the inhibition of the synthesis of nodulation factors. Then, whenever some bacteria retaining the plasmid with the *gdhA* gene are able to infect the bean root, perhaps aided in *trans* by a considerable population of bacteria that have lost the plasmid, they severely impair the nodule development and nodule physiology (Bravo et al. 1988, and this paper). The alteration of one or both of these two processes may explain the effect of the *ntrC*$^-$ mutant strain in restoring nodulation and nitrogen fixation, when it expresses GDH (CFN2012/pAM1a) (Table 4). Other differences in the nitrogen metabolic status of the wild-type strain as compared to the *ntrC*$^-$ mutant strain, in spite of the similar GOGAT activities and amino acid concentrations observed (Tables 2 and 3), that may influence the selective loss of the pAM1a plasmid only from the wild type and not from the *ntrC*$^-$ mutant during symbiosis, cannot be excluded.

Data at hand indicate that the expression of GDH is compatible with nodulation and nitrogen fixation during symbiosis, in an *ntrC*$^-$ mutant background of the microsymbiont (Tables 2 and 4). One approach to test this interpretation would be to study the symbiotic effect of the expression of GDH driven by a *Rhizobium nif* gene promoter, which is known to be expressed only during nitrogen fixation in the bacteroid, both in the wild type or *ntrC*$^-$ background; we are currently doing these studies. Another approach would be to add the Nod factors, as has recently been reported (Relic et al. 1994), when inoculating beans with a low inoculum of the CFN42/pAM1a strain.

MATERIALS AND METHODS

Bacterial strains, plasmids, and cultures.

The bacterial strains and plasmids used are listed in Table 1. *E. coli* strains were grown at 37°C on either solid, or liquid LB medium (Miller 1972). Batch liquid cultures of *R. etli* were grown on minimal medium (MM) (Beringer 1974) with either 10 mM NH_4Cl or 10 mM glutamate as the nitrogen source and 10 mM succinate as the carbon source, at 30°C and shaken at 200 rpm. They were inoculated with bacteria grown overnight on PY (Bravo and Mora 1988) liquid medium cultures, to a concentration of 0.05 OD_{540}. When it was required, media were supplemented with the appropriate antibiotics as follows (mg/liter): (50), kanamycin (Km) (50), nalidixic acid (Nal) (20), chloramphenicol (Cm) (10), and tetracycline (Tc) (10). Plasmids were conjugated to different *R. etli* receptor strains by triparental mating using pRK2013 as a helper plasmid. To obtain the DEM301, strain homogenotization was done by triparental mating of the CFN2012, *ntrC*$^-$ mutant (Moreno et al. 1992), receptor strain, plasmid, and *E. coli* bearing pRp30 (Vázquez et al. 1991) and selecting for Cm^rTc^s colonies. The plasmid stability in *R. etli* liquid cultures was determined by replicating the colonies grown in solid PYNal rich medium inoculated from each liquid cultures in PYTc plates and determining the percentage of Nal^r Tc^r colonies.

Construction of pAM1a plasmid.

The pSAE4 plasmid (Sánchez-Pescador et al. 1982) contains the complete GDH coding sequence plus 170 bp from the 5′ upstream regulatory region and 450 bp from the 3′ downstream region of the *E. coli gdhA* gene (Valle et al. 1984) in a 1.5-kb *Dde*I–*Dde*I fragment. This *Dde*I–*Dde*I fragment from the pSAE4 plasmid was purified, filled-in with Klenow and cloned into the previously filled-in *Kpn*I site of pMW157 (Williams et al. 1988), thus generating the intermediate plasmid: pAM300. The pMW157 plasmid (Williams et al. 1988), contains the 1.1-kb T1T2 transcription-translation terminator and the Ω (Sm^r/Sp^r) fragment (Prentki and Krisch 1984) cloned into the *Sma*I and *Eco*RI sites, respectively, of the polylinker from plasmid pIC20H (Marsh et al. 1984). The pAM300 intermediate plasmid was cleaved with *Xba*I and a 4.8-kb fragment containing the *gdhA* gene flanked by the two transcription-translation terminators was purified. This fragment was ligated separately to pTR101 (Weinstein et al. 1992), previously linearized with *Xba*I, thus generating the pAM1a plasmid (Fig. 1).

Determination of GDH and GOGAT enzyme activities.

Enzyme activities were assayed in cell-free extracts of *R. etli* strains grown for 10 or 24 h on MM batch cultures. The GDH and GOGAT activities were determined as reported (Bravo et al. 1988). The GOGAT activity was determined from cell-free extracts previously desalted through Sephadex G25. A control enzyme assay containing 50 mM L-methionine sulfone was performed in every case and whenever a residual activity, due to the presence of an active GDH, was observed in this control it was subtracted from the GOGAT activity present in the enzyme assay performed without the specific GOGAT inhibitor. One unit (U) of either GDH or GOGAT specific activities represents 1 nmol NADP/min/mg protein.

Amino acid determination.

Crude extract preparations for intracellular amino acid determinations were obtained in 80% ethanol, for extracellular amino acid determinations 20 ml of culture media was centrifuged and the supernatant, free of bacteria, was lyophilized. Amino acids were separated and quantified using high-performance liquid chromatography (HPLC) reverse-phase with a Nova-Pack C18 (3.9 × 150 mm) column (Waters). They are expressed as μmol/mg protein.

Plant inoculation.

Phaseolus vulgaris cv. Negro Jamapa seedlings were surface sterilized and germinated as previously reported (Bravo et al. 1988). The *R. etli* strains used as inoculum were grown overnight on PY medium, washed twice with 0.85% NaCl, and diluted to 0.005 OD_{540}. Each germinated seedling was inoculated with 1 ml of bacterial suspension. This inoculum concentration corresponds to 10^7 cells/plant. Greenhouse growth conditions and plant maintenance have been reported

(Bravo et al. 1988). To evaluate the symbiotic phenotype of the different *R. etli* strains used, 25 plants were analyzed at 18 or at 25 days postinoculation for each strain. The nodule dry weight, nitrogenase activity, and plant dry weight were determined in every plant and the media and standard deviation (SD) were obtained for each parameter. To determine the proportion of bacteria with or without plasmids, that were present in the nodules of plants inoculated with different *R. etli* strains, nodules were detached from the roots, surface sterilized by soaking them in 20% commercial bleach for 5 min and washed three times in sterile distilled water. Then the nodules were dissected with a sterile scalpel and the bacteria released were resuspended in 1 ml of distilled water, diluted, and plated into PYNal rich medium plates. After colonies appeared they were replicated in PYTc plates for the wild-type strains or PYKmTc plates for the *ntrC*$^-$ strain. The percentage of Tc^r colonies represent those that have retained the plasmid.

Determination of nitrogenase activity.

Nitrogenase activity was determined in detached, nodulated roots by the acetylene reduction method. Bean plants were harvested and the root system was immediately detached and incubated with 1/80 (v/v) acetylene. The ethylene produced at different time points was quantified using a Varian 3300 gas chromatograph. The nitrogenase specific activity is expressed as μmol ethylene/h/g nodule dry weight.

Preparation of root exudate.

Root exudates from *P. vulgaris* cv. Negro Jamapa plant culture were prepared as follows. Seeds were surface sterilized by soaking them in ethanol for 1 min and then in 25% commercial bleach for 15 min, followed by six rinses with sterile distilled water. Seeds were germinated on moist sterile filter paper for 2 days and transferred to a plastic net located above 50 ml of deposit-free liquid Jensen medium (Vincent 1970) in sterile joined Magenta vessels. The cultures were incubated for 5 days with a 16-h photoperiod provided by cool-white fluorescent light (54 μmol/m^{-2}/s^{-1}) at 25°C, the roots were shielded from light with foil. The liquid medium from each plant culture was collected and centrifuged aseptically, and it was concentrated 10-fold by lyophilization. When mentioned in the text, bean root exudate was isolated after inoculation either with the CFN42/pTR101 or the CFN42/pAM1a strain that was added to the liquid medium when the plant culture was initiated. The *R. etli* strain used to inoculate these cultures was grown and washed as previously mentioned. The inoculum concentration used was 10^7 cells/plant.

Bioassay for *nod* gene inducing activity.

R. etli UBP102 or DEM301 strains harboring the pTR101 or the pAM1a plasmids (Table 1) were grown in solid YMB medium (Hooykaas et al. 1977) containing Cm and Tc and single colonies were used to inoculate liquid precultures of MM (Beringer 1974) with different ammonium concentrations (from 0.2 to 30 mM ammonium) as nitrogen source, which were grown for 48 h. Cells from these precultures were diluted 0.05 to OD_{540} in 4.5 ml of the same medium in the presence of 0.5 ml of concentrated bean root exudate or 1.2 μM naringenin, and were incubated for 6 h shaken at 30°C. Cultures incubated in the absence of inducer were used as controls. The β-galactosidase activity assays were carried out as reported (Vázquez et al. 1991). One unit (U) of β-galactosidase specific activity represents 1 nmol ONPG/min/mg protein. The β-galactosidase activity was determined from at least four different culture replicas of each strain, in each condition. The percentage of β-galactosidase induction was calculated after subtracting the basal β-galactosidase activity present in the control cultures (without inducer) of each condition.

Determination of Nod factor production.

The Nod factors produced and excreted by different *R. etli* strains were determined by thin-layer chromatography. One-milliliter bacterial cultures in MM were labeled with 0.2 μCi C^{14}-glucosamine (52.6 μCi/mmol, Amersham). The flavonoid used to induce the Nod metabolite production was naringenin 1.2 μM. Bacterial growth was estimated by OD_{600}, and final volumes to be processed were adjusted accordingly to consider similar amounts of bacteria. Cultures were centrifuged and supernatants were chromatographed through C-18 Sep-Pak cartridges as described (Poupot et al. 1995). The methanol fraction were recovered, dried under nitrogen, and used for high-performance thin-layer chromatography (HPTLC) on Silica 60 plates in direct-phase using chloroform, methanol and 5 N ammonia (3:3:1, v/v). Hyperfilm β max was used for autoradiography.

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