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NifA-Dependent Expression of Glutamate Dehydrogenase in *Rhizobium etli* Modifies Nitrogen Partitioning During Symbiosis

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Constitutive expression of foreign glutamate dehydrogenase in *Rhizobium etli* inhibits bean plant nodulation (A. Mendoza, A. Leija, E. Martínez-Romero, G. Hernández, and J. Mora. Mol. Plant-Microbe Interact. 8:584-592, 1995). Here we report that this inhibition is overcome when controlling *gdhA* expression by NifA, thus delaying the GDH activity onset after nodule establishment. Expression of *gdhA* modifies the nitrogen partitioning inside the bacteroid, where newly synthesized ammonia is preferentially incorporated into the amino acid pool instead of being exported to the infected cells. As a consequence, the fixed nitrogen transport to the leaves, measured as the ureides content in xylem sap, is significantly reduced. Nitrogenase activity, although not *nifHDK* expression, is significantly reduced in bacteroids expressing *gdhA*, probably due to the utilization of energy and reducing power for nitrogen assimilation. Here we show that ammonia assimilation inside *R. etli* bacteroids is active, albeit at low levels, and when enhanced is deleterious to the symbiotic performance. This leads us to believe that further reduction of the basal nitrogen metabolism in the bacteroid might stimulate the nitrogenase activity and increase the nitrogen supply to the plant.

Additional keywords: *nifHc* promoter, nitrogen fixation.

Virtually all free-living, nitrogen-fixing bacteria assimilate ammonium diazotrophically through the glutamine synthetase (GS)-glutamate synthase (GOGAT) pathway instead of the glutamate dehydrogenase (GDH)–GS pathway (Bravo and Mora 1988; Carlson et al. 1987). Low levels of assimilatory GDH activity have been found in several *Rhizobium* spp. (Brown and Dilworth 1975; Ali et al. 1981; Fottrell and Mooney 1969; Osburne and Signer 1980), although this activity is low compared with GOGAT activity (Brown and Dilworth 1975; O'Gara et al. 1984). *Rhizobium etli* CFN42 does not contain a functional NADPH-dependent GDH and relies completely on the GS-GOGAT pathway for ammonium assimilation in free life and during symbiosis (Bravo and Mora 1988). The GS-GOGAT pathway has a higher affinity for ammonium than the GDH-GS pathway, thus allowing efficient assimilation of the limited amounts of ammonia produced by nitrogenase.

Consistent with the absence of a significant activity of GDH in *Rhizobium* and *Bradyrhizobium* all the GOGAT mutants isolated so far are glutamate auxotrophs (Donald and Ludwig 1984; Kondorosi et al. 1977; Ludwig 1978; O'Gara et al. 1984; Osburne and Signer 1980). We previously reported that *R. etli* (formerly *Rhizobium leguminosarum* bv. *phaseoli*) (Segovia et al. 1993) assimilates ammonia through the GS-GOGAT pathway and that no GDH activity could be detected (Bravo and Mora 1988). *Rhizobium* spp. contain two GS isozymes, but only one of them (GSI) is active during symbiosis (Moreno et al. 1991). GSII activity is not present in *R. etli* (Moreno et al. 1991) nor in *R. meliloti* bacteroids (de Bruijn et al. 1989). Also, neither the transcription of a *glnII-lac*Z fusion (de Bruijn et al. 1989) nor the presence of the GSII protein (screened with anti-GSII-specific antiserum) could be detected (Shatters et al. 1989). In contrast, GOGAT activity has been found in bacteroids isolated from nodules elicited by different *Rhizobium* spp. (Brown and Dilworth 1975).

Mutants of *R. meliloti* lacking GOGAT are symbiotically proficient (Osburne and Signer 1980) whereas a *Bradyrhizobium japonicum* GOGAT$^-$ is fix$^-$ (O'Gara et al. 1984). The abolishment of nitrogen fixation in mutant strains lacking GS depends on the specific symbiosis tested. In *R. meliloti,* mutants lacking both GSI and GSII are symbiotically competent (de Bruijn et al. 1989; Shatters et al. 1989). A third GS isozyme has been observed in both *R. meliloti* (Shatters et al. 1993) and *R. etli* (Espín et al. 1990), but its role in symbiosis is unknown. A *B. japonicum* mutant lacking both GSI and GSII is severely impaired, both for nodulation and nitrogen fixation (Carlson et al. 1987), and no GSIII activity has been reported.

The general acceptance that *Rhizobium* does not assimilate ammonia during symbiosis has prevented a detailed study of the active bacterial nitrogen metabolism in planta. There is abundant evidence indicating that bacteroids synthesize different nitrogen-containing metabolites during symbiosis (Salminen and Streeter 1987). Soybean bacteroids synthesize extensive amounts of glutamate, and deamination of aspartate by aspartase stimulates both nitrogen fixation and respiration in

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soybean bacteroids (Kouchi et al. 1991). Also, an *R. meliloti* mutant lacking aspartate amino transferase is impaired in nitrogen fixation (Rastogi and Watson 1991).

We are interested in modifying the ammonium assimilation in *R. etli* to evaluate and enhance its symbiotic performance. In our first attempt we introduced the *gdhA* gene from *Escherichia coli* expressed under the control of its own constitutive promoter into *R. etli*, and showed that this activity has a strong negative effect in the symbiosis (Bravo et al. 1988). Expression of the *E. coli gdhA* was correlated with the formation of ineffective nodules mixed with a large number of effective nodules formed by bacteria that had lost the *gdhA*-harboring plasmid (Bravo et al. 1988).

To eliminate the production of mixed-nodule population and to assess the role of GDH during nodule development, the same *gdhA* gene was inserted into the stable vector pTR101 (Weinstein et al. 1992) and introduced into *R. etli* cells. A total inhibition of bean nodulation was observed when the minimal effective inoculum of *R. etli* was used. This inhibition is due to the inability of bean-root exudate compounds to induce *nodA* expression in the presence of GDH in an NtrC-dependent manner (Mendoza et al. 1995). Our interpretation is that expression of GDH strongly down-regulates *nodA* and consequently decreases nodulation, when a high intracellular amino-nitrogen pool accumulates due to the constitutive activity of GDH prior to or during nodule formation (Mendoza et al. 1995).

To restrict the onset of GDH activity until nodule formation and determine the effect of this delayed expression on *R. etli* bacteroids, we report the NifA-dependent expression of *gdhA* and evaluate the consequences on the nitrogen metabolism in the bacteroid.

RESULTS

Construction and analysis of the *nifHc-gdhA* fusion.

Delaying expression of GDH until after nodule establishment would provide an opportunity to evaluate the effect of modifying the bacteroid ammonia assimilation pathway. *R. etli* contains three copies of *nifH* that code for the nitrogenase reductase (Quinto et al. 1985). One of them, *nifH*c, is expressed at higher levels than the other two and is induced during nodule development in an NifA-dependent manner (Valderrama et al. 1996). Its regulatory region contains an unusual NifA-binding site upstream of the RpoN-dependent promoter (Valderrama et al. 1996). To construct a NifA-dependent *gdhA* gene, a promoterless *gdhA* from *E. coli* was inserted downstream from the *nifH*c promoter in pTR101, resulting in plasmid pAM340 (see Materials and Methods and Figure 1).

The expression of NifA-regulated genes in *Rhizobium* is oxygen sensitive (Fischer 1994). To assess the NifA-dependent expression of the *nifHc-gdhA* fusion contained in pAM340, we determined the GDH activity of the *R. etli* wild-type strain CFN42 harboring this plasmid when grown under different oxygen concentrations. As shown in Table 1, GDH activity is specifically induced in cultures grown under 1% oxygen, compared with cultures grown under atmospheric concentrations, a known physiological condition for the NifA-mediated induction of *nifH*c (Valderrama et al. 1996). In contrast, expression of *gdhA* from its constitutive promoter was independent of the oxygen concentration and lower than that induced by NifA in the CFN42/pAM340 strain (Mendoza et al. 1995).

Symbiotic efficiency of *R. etli* harboring the pAM340 plasmid.

Symbiotic expression of *nifH*c is induced at 10 to 13 days postinoculation (dpi) of bean roots, once the nodules are completely structured (Valderrama et al. 1996). In accordance, we found that bacteroids harboring the pAM340 plasmid induce GDH activity from 23 units at 13 dpi to 317 units at 18 dpi, when bean nodules are fully developed and fixing nitrogen. Delaying GDH expression allows normal nodulation to occur (Fig. 2A). However, nitrogenase activity in plants inoculated

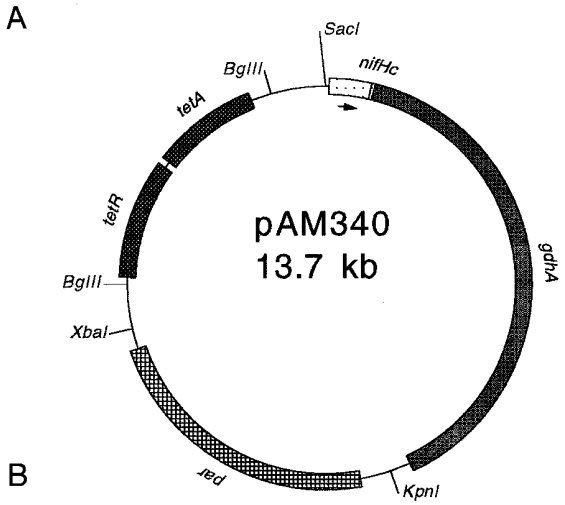

Fig. 1. Physical map of plasmid pAM340 carrying the *nifHc-gdhA* fusion and nucleotide sequence of the regulatory region. **A,** Restriction map of recombinant plasmid pAM340 (drawn to scale). Arrow indicates relevant fragment sequenced and presented in **B**. See Materials and Methods for construction strategy. **B,** Nucleotide sequence of non-translated 5′ region of the *nifHc-gdhA* fusion. Regulatory motifs are underlined: NifA-binding site (TGG-N10-ACA); RpoN-dependent promoter (GG-N10-GA); and ribosome-binding site (TAAGG). Junction is presented between dashes, where only the first nucleotide of the *nifHc* ATG translation start codon is retained. The *gdhA* MET start codon is in bold.

Table 1. Expression of pr.*nifHc-gdhA* fusion in *Rhizobium etli* CFN42 cultures grown at low and high oxygen concentrations[a]

Plasmid	Low O_2 (1%)	High O_2 (20%)
pAM1a	195 (± 24.1)	227 (± 11.3)
pAM340	410 (± 42.4)	23 (± 4.2)

[a] Glutamate dehydrogenase (GDH) activity is expressed as nmol of oxidized NADPH per min per mg of protein. Values are means of two independent determinations with two samples per assay (n = 4). Numbers in parentheses represent ± standard deviations.

with the CFN42/pAM340 strain was statistically reduced ($P < 0.05$) from that of the CFN42/pAM341 strain by 11, 21, and 49% at 18, 25, and 32 dpi, respectively (Fig. 2B). As a probable consequence of the lower nitrogenase activity, total yield of plants inoculated with strain CFN42/pAM340 was reduced by 30% after 32 dpi compared with those inoculated with strain CFN42/pAM341 (Fig. 2C).

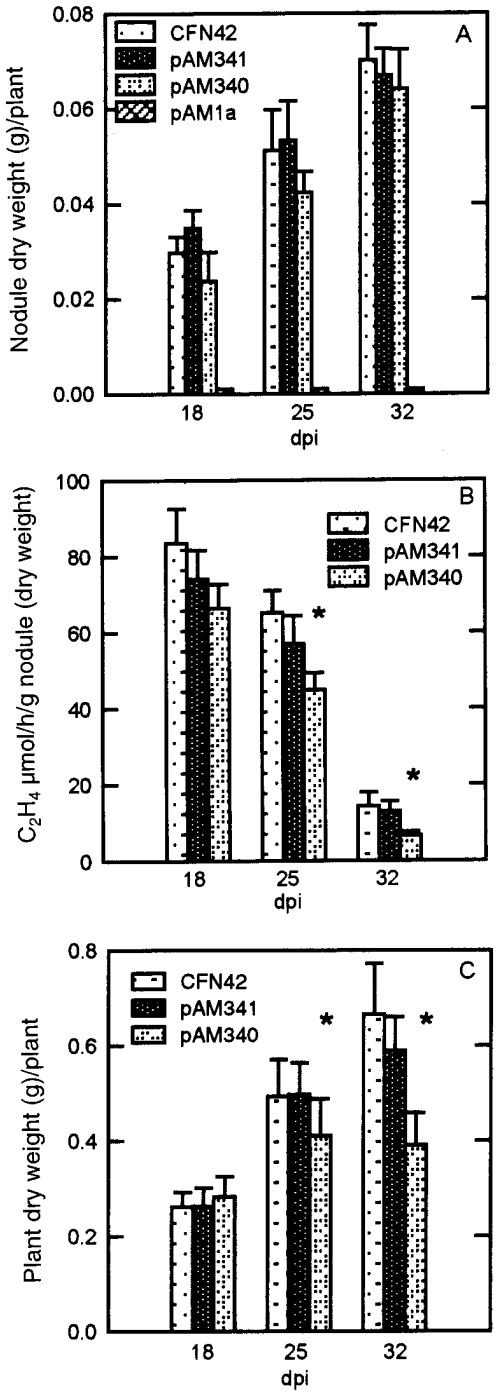

Fig. 2. Symbiotic effects as result of GDH expression under the *nifHc* promoter in *Rhizobium etli*. Nodulation (**A**), nitrogenase specific activities (**B**), and plant dry weights (**C**) of *Phaseolus vulgaris* inoculated with CFN42, CFN42/pAM341, and CFN42/pAM340 strains. Specific nitrogenase activities are expressed as µmol of ethylene produce per h per g of dry weight nodule. * indicates that the means of the samples are different at the 0.05 level ($P < 0.05$). Values are means ± standard deviations from three independent experiments with 8 plants for each condition and time (n = 24).

One of the more dramatic effects of the lower nitrogenase activity is the significant reduction of the total plant nitrogen content by an average of 20 ± 2% during the symbiosis in plants inoculated by CFN42/pAM340, compared with CFN42/pAM341. In one experiment plants were grown until seeds could be harvested (90 days) and it was noteworthy that the total nitrogen content of seeds from CFN42/pAM340-inoculated plants was significantly reduced (51.5 mg of seed nitrogen per plant), compared with plants inoculated with CFN42/pAM341 (70.3 mg of seed nitrogen per plant). More than 99% of the bacteroids isolated from nodules retained the pAM340 or pAM341 plasmids after 25 or 32 dpi, therefore controlled expression of *gdhA* avoids the strong selection pressure against the plasmid, as was previously suggested with pAM1a (Fig. 2A; Mendoza et al. 1995).

Amino acid concentration in bacteroids of *R. etli* expressing GDH activity.

Timed expression of GDH activity coincides with the onset of nitrogen fixation. As a result, there is a 2.3-fold increase in the glutamate content and a 2.2-fold increase in the amino-nitrogen content of bacteroids isolated at 25 dpi from bean roots inoculated with the CFN42/pAM340 strain, compared with the CFN42/pAM341 strain (Table 2).

Ureide content in xylem sap.

In bean plants, fixed nitrogen is transported to bean leaves as ureides, primarily allantoin and allantoic acid (Schubert 1986). NifA-dependent expression of GDH in strain CFN42/pAM340 reduced the ureide content in the xylem sap by 30% at 18 dpi and by 12% at 25 dpi, compared with that of the CFN42/pAM341 strain (Table 3).

Histology of bean root nodules expressing GDH.

Bean roots were harvested at 18 and 32 dpi from plants inoculated with the CFN42/pAM340 strain or with the wild-type CFN42 strain. Longitudinal sections of some of the nodules formed by the CFN42/pAM340 strain showed disruption of internal tissue (compare Fig. 3A and B). The ultrastructure of these damaged sections indicates a partial loss or degradation of the infected cell wall and bacteroid deformations (Fig. 3D). At 18 dpi, 10% of the nodules formed by the CFN42/pAM340 strain were damaged, whereas after 32 dpi 90% of the nodules were disrupted. Plants inoculated with the CFN42/pAM341 or the wild-type CFN42 strains showed no more than 10% of damaged nodules at any time.

Table 2. Free amino acid concentrations in *Rhizobium etli* CFN42 bacteroids expressing the pr.*nifHc-gdhA* fusion[a]

Strain	Glutamate	Other amino acids[b]	Amino-nitrogen[c]
CFN42	72.9 (± 7.6)	36.8 (± 8.4)	109.7 (±16.1)
pAM341	77.5 (± 9.4)	62.5 (± 12.4)	140.1 (± 21.7)
pAM340	179.8 (± 14.1)	174.3 (± 16.2)	353.9 (± 30.0)

[a] Concentrations are expressed as nmol per mg of protein. Determined at 25 days postinoculation. Data presented are the mean of two independent experiments with two samples per assay (n = 4). Numbers in parentheses represent ± standard deviations.

[b] Other amino acids: aspartate, threonine, serine, glutamine, glycine, and alanine.

[c] The sum of amino-nitrogen from glutamate plus other amino acids.

DISCUSSION

In contrast to what has been observed in *Bradyrhizobium* and *Azorhizobium*, where GOGAT$^-$ strains are defective in symbiotic nitrogen fixation (O'Gara et al 1984; Hilgert et al. 1987), *Rhizobium* mutants lacking the ammonia assimilation enzymes GOGAT or GS are symbiotically proficient (Osburne and Signer 1980; de Bruijn et al. 1989; Shatters et al. 1989). These observations lead us to believe that during symbiosis between *Rhizobium* and leguminous plants, all the dinitrogen fixed is delivered to the plant as ammonium. However, there is a lack of direct information regarding how much, if any, the bacteroid participates in assimilating the dinitrogen fixed.

We have explored the role of the bacteroid ammonia assimilation during symbiosis, studied by constitutively expressing the *E. coli gdhA* in *Rhizobium etli* (Mendoza et al. 1995), and found that nodulation was impaired. The results of Mendoza et al. (1995) suggested that the accumulation of organic nitrogen repressed the synthesis of *nod* factors in an NtrC-dependent manner. These data indicated that the nodulation induced by *R. etli* occurs in an environment deprived of reduced nitrogen.

To bypass the effect of the apparent nitrogen repression over the nodulation process, we placed control of *gdhA* expression in *R. etli* under NifA. In this way it should be possible to determine if the ammonia synthesized by nitrogenase is available for assimilation to *R. etli* bacteroids and, if so, whether this assimilation influences nitrogen fixation or nitrogen partitioning between the bacteria and the plant. This was accomplished by placing a promoterless *gdhA* gene under the control of the strong *R. etli nifH*c promoter (Valderrama et al. 1996). Our results show that the expression of GDH from the pr.*nifHc-gdhA* (pAM340) depends on a low oxygen concentration in culture, a known condition for NifA-dependent induction (Table 1). The nitrogenase genes in *R. etli* are induced only 10 to 13 days after inoculation onto bean roots (Valderrama et al. 1996). Consequently, expression of the pr.*nifHc-gdhA* construction is delayed up to this time, thus allowing nodulation prior to GDH expression (Fig. 2A).

Here we report that expression of GDH after nodule establishment modifies nitrogen partitioning inside the bacteroid. GDH captures most of the newly synthesized ammonia into the amino acid pool (Table 2). As a consequence, the fixed nitrogen is not exported to the infected cells and the ureides transport is reduced (Table 3). Nitrogenase activity, although not *nifHDK* expression, is significantly reduced in bacteroids expressing *gdhA* (Fig. 2B), probably due to an increase in ATP and NADPH consumption of the ammonia assimilation enzymes, therefore diverting the energy pool from being used for other processes. Reduction of the nitrogen supply to the plant modifies the nutritional status of the leaves, which might react, reducing the photosynthate transport into infected cells, intensifying the metabolite imbalance, and triggering premature senescence of some nodules (Fig. 3B).

Recently, it has been shown that alfalfa inoculated with *R. meliloti glnB* mutants is nitrogen starved in the absence of added combined nitrogen (Arcondeguy et al. 1997), and it was hypothesized that P(II) controls the expression or activity of a bacteroid ammonium transporter. Therefore, the P(II) protein affects both *Rhizobium* nitrogen metabolism and alfalfa nodule development.

To our knowledge, this is the first report of selective modification of nitrogen partitioning during the legume-*Rhizobium* symbiosis by expression of a foreign enzyme. This modification of bacteroidal nitrogen assimilation has a negative impact on the nitrogen translocation to the plant. The strongly negative effect of GDH on symbiosis could be the selective pressure leading to loss of this enzyme in *R. etli*.

Based on the results presented here, we propose that a GOGAT mutant of *R. etli* would result in an altered nitrogen partitioning between the plant and the bacteria, since the absence of GOGAT would preclude bacteroid ammonia assimilation. Preliminary observations by our group lead us to believe that, in the absence of GOGAT, excess of ammonia could be alternatively exported to the infected cell, where it could be assimilated by the plant GS-GOGAT pathway, therefore increasing the nitrogen availability for ureides synthesis.

Table 3. Ureide concentration in the xylem sap of *Phaseolus vulgaris* inoculated with *Rhizobium etli* CFN42 with or without *gdhA*

Plasmid	dpi[a]	Ureide (mM)
None	18	0.358 (± 0.05)
	25	0.447 (± 0.05)
pAM341 (pr.*nifHc*)	18	0.305 (± 0.06)[b]
	25	0.404 (± 0.05)[c]
pAM340 (pr.*nifHc*::*gdhA*)	18	0.218 (± 0.03)[b]
	25	0.367 (± 0.04)[c]

[a] Days postinoculation. Data presented are the mean of total (n = 5) samples from two independent experiments. Each sample is a pool from 5 plants. Numbers in parentheses represent ± standard deviations.
[b] With *t* test analysis, this pair of values differs, with a level of confidence ($P < 0.05$).
[c] With *t* test analysis, this pair of values differs, with a level of confidence ($P < 0.05$).

MATERIALS AND METHODS

Bacterial strains, plasmids, and media.

Plasmids and strains used in this work are described in Table 4. *E. coli* strains were grown at 37°C in LB complex medium (Miller 1972). *R. etli* strains were grown at 30°C in PY complex medium or in minimal medium containing 10 mM NH_4Cl and 10 mM succinate as nitrogen and carbon sources (Encarnación et al. 1995). Cultures in minimal medium were inoculated to an $A_{540} = 0.05$ with bacteria grown overnight on PY medium and shaken at 200 rpm (Controlled Enviroment Incubator Shaker, New Brunswick Scientific, Edison, NJ) at 30°C. Antibiotics were added in the following final concentrations: 50 µg of kanamycin per ml, 20 µg of nalidixic acid per ml, 100 µg of carbenicillin per ml, and 10 µg of tetracycline per ml. Plasmids were conjugated into *R. etli* in a triparental mating, with pRK2013 as a helper plasmid.

DNA manipulations and sequencing.

All DNA manipulations and *E. coli* transformations were performed following standard procedures (Sambrook et al. 1989). DNA fragments were purified from agarose gels with the GeneClean Kit (Bio101, Buena Vista, CA). The sequence of the *nifHc* promoter fused to the *E. coli gdhA* was performed with the oligonucleotide (5′-CCGATCTGGCAAGGCTCT ACGAC-3′) by the dideoxy method (Sanger et al. 1977) with the Thermo Sequenase Dye Terminator Cycle Sequencing premix (Amersham LIFE SCIENCE, Cleveland, OH) and DNA

Sequencing Dye Terminator Cycle Sequencing ready reaction (Perkin-Elmer, Forest City, CA).

Construction of pAM340 and pAM341 plasmids.

The promoterless *E. coli gdhA* gene (Valle et al. 1984) was obtained from the pSAE4 plasmid (Sánchez-Pescador et al. 1982) as a 1.7-kb *Vsp*I-*Dde*I fragment, blunt-ended, and cloned into the *Sma*I site of pUX19 to create pAM320. A 1.7-kb *Eco*RI-*Sac*II fragment from pAM320 was then subcloned into pBlueScript II KS (Stratagene, La Jolla, CA) to create pAM321. To generate a *nifHc-gdhA* fusion, the promoter of the *R. etli* CFN42 *nifHc* gene from the pCQ23 was subcloned

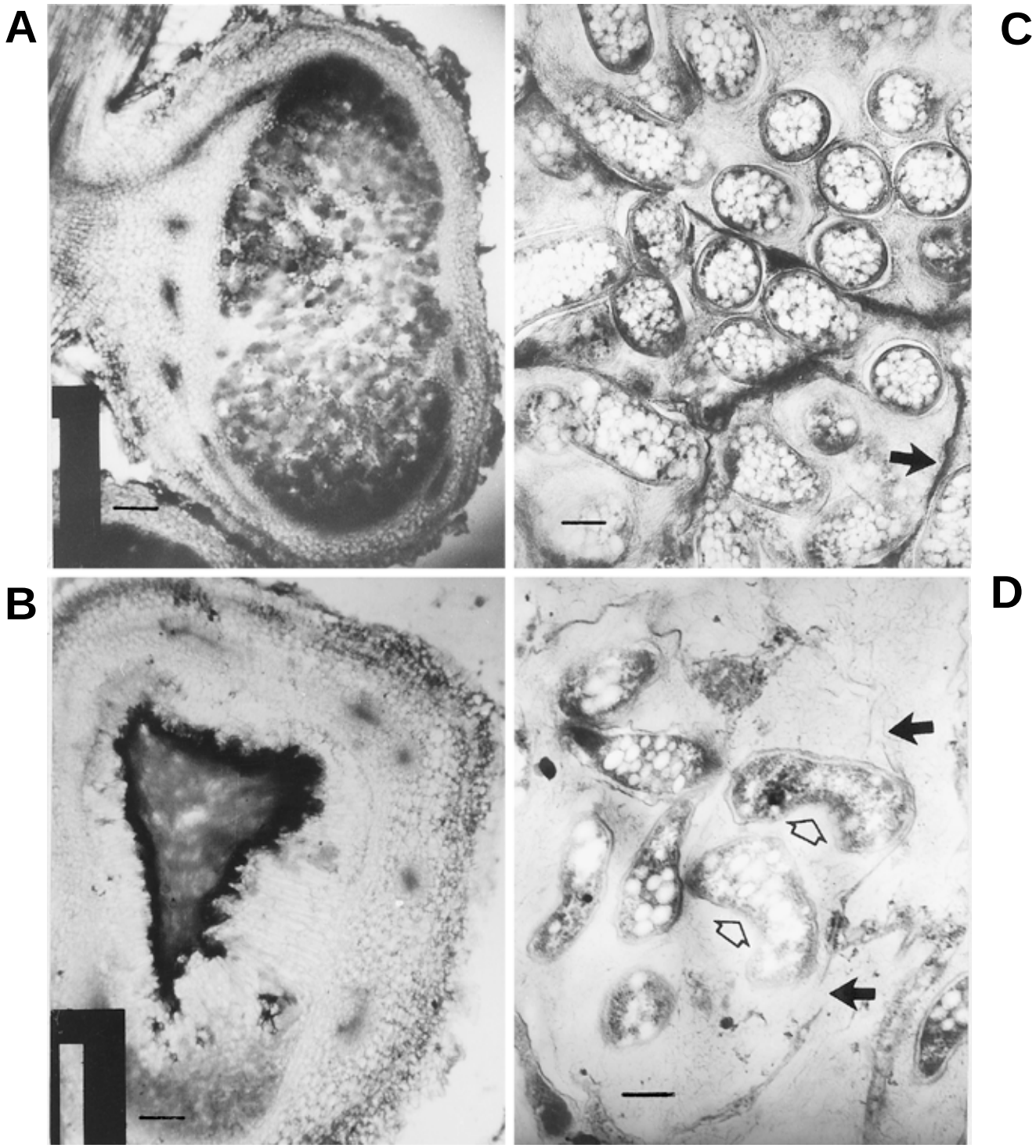

Fig. 3. Histological condition of nodules infected by *Rhizobium etli* /pAM340. **A,** Longitudinal section of a wild-type nodule showing normal tissue. Bar is 100 µm. **B,** Longitudinal section of a nodule formed by a plant inoculated with CFN42/ pAM340. Internal nodule necrosis is shown. Bar is 100 µm. **C,** Transmission electron micrograph (TEM) of a wild-type nodule; arrow indicates cell wall. Bar is 0.6 µm. **D,** TEM of nodule formed by a plant inoculated with CFN42/pAM340. Filled arrows indicate disruption on cell wall; open arrows point to bacteroids deformation. Bar is 0.4 µm.

as a 1.5-kb *Eco*RI-*Hga*I fragment, blunt-ended, and ligated into the unique *Eco*RV site of pAM321 to produce pAM322. The pr.*nifHc-gdhA* fusion was subcloned as a 3.2-kb *Kpn*I-*Sac*I fragment into the stably inherited vector pTR101 (Weinstein et al. 1992) to create pAM340 (Fig. 1). To create pAM341, the 1.5-kb *Eco*RI-*HgaI* fragment containing the *nifHc* promoter was directly subcloned into pTR101. Nucleotide sequence of the recombinant pAM340 plasmid is shown in Figure 1.

Determination of GDH activity.

Cells from exponentially growing cultures of *R. etli* in PY medium were collected, washed, and resuspended in 10 ml of minimal medium; 150-ml bottles containing 50 ml of minimal medium were inoculated at an initial A_{540} = 0.05, sealed with rubber stoppers, and immediately flushed with several volumes of a 1% oxygen–99% argon mixture (analytical grade; Linde, México City, México). Bottles for 20% oxygen cultures were shaken under atmospheric conditions. After 12 h of incubation at 200 rpm (Controlled Enviroment Incubator Shaker, New Brunswick Scientific) and at 30°C, cells were collected to determine GDH-specific activity as previously reported (Bravo and Mora 1988). Specific activities are reported as nmoles of oxidized NADPH per minute per mg of protein.

Nodulation test, nitrogenase activity, and nitrogen content in bean plants.

Phaseolus vulgaris cv. Negro Jamapa seedlings were surface sterilized and germinated as previously reported (Bravo et al. 1988). *R. etli* strains used as inoculum were grown overnight on PY medium, washed twice with 0.8% NaCl, and diluted to an A_{540} = 0.05. Germinated seedlings were planted in groups of five in thoroughly autoclaved pots containing vermiculite as support, then each one was inoculated with 1 ml of bacterial resuspension (approximately 10^7 cells per plant). Each experiment included noninoculated control plants with and without added nitrogen and samples of them were simultaneously harvested at the same time as the experiment. When any of the noninoculated control plants were nodulated, the whole experiment was discarded.

Greenhouse conditions were as follows: temperature from 22 to 28°C and relative humidity from 50 to 60%. Groups of 8 plants grown under each condition were harvested at 18, 25, and 32 dpi, and their nodule dry weight, nitrogenase activity, total plant dry weight, and nitrogen content were determined for every plant, including the control plants. Bacteria were isolated from nodules and their identity verified by their antibiotic resistance pattern. Nitrogenase specific activity (µmol ethylene generated per h per g of nodule dry weight) was determined by incubating the detached root with 1/80 (vol/vol) acetylene. Ethylene production was estimated with a Varian 3300 gas chromatograph. Total nitrogen content of samples from dry plants was determined with a nitrogen analyzer (model ANTEK 7000; Antek Instruments, Houston, TX) and is reported as mg of nitrogen per mg of dry plant or powdered seed. Statistical analysis were performed according to Steel and Torrie (1980).

Purification of bacteroids.

Fresh nodules (5 g) were detached from the roots of several plants inoculated with the same strain, crushed in phosphate-buffered saline (150 mM NaCl, 50 mM KH_2PO_4, pH 7.6), and filtered through four layers of cheesecloth. Bacteroids were isolated by centrifugation through self-generated Percoll gradients (Reibach et al. 1981). Purified bacteroids were assayed immediately for GDH activity and amino acid content.

Determination of free amino acids.

Free amino acids were quantified fluorimetrically by a precolumn derivation technique with 9-fluroenylmethyl-chloroformate and Nova-Pack C18 column (Waters, Milford, MA; 3.9 × 150 mm inside diameter) according to Cevallos et al. (1996). Amino acid separation was performed with a 20 to 73% elution gradient of solvents A and B (solvent A, 0.05 M sodium acetate [pH 3.5 to 3.8] adjusted with acetic acid; solvent B, acetonitrile) at a flow rate of 1 ml/min at 45°C. Amino acids were detected with a fluorometer (Waters model 420 AC) set at excitation and emission wavelengths of 254 and 313 nm, respectively, and equipped with a G475 Hg lamp (Waters). Amino acid concentrations are expressed as nmol per mg of protein.

Table 4. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
Rhizobium etli		
CFN42	Wild-type strain; Sm^rNal^r	Bravo and Mora 1988
Escherichia coli		
HB101	F^- *hsd S20-recA13*	Boyer and Roulland-Dussoix 1969
Plasmids		
pRK2013	Col E1 *mob+Tra+*; Km^r	Figurski and Helinski 1979
pSAE4	pBR322, 3.6-kb *Hind*III-*Pst*I fragment containing the *E. coli gdhA* gene	Sánchez-Pescador et al. 1982
pCQ23	pBR328, 4.2-kb *Eco*RI fragment containing the *R. etli nifH*c gene	Quinto et al. 1985
pTR101	pTR100 (mini-RK2) vector, with 0.8-kb stability locus; Tc^r	Weinstein et al. 1992
pUX19	Cloning vector, Km^r	Vieira et al. 1991
pAM1a	pTR101/*E. coli gdhA* gene	Mendoza et al. 1995
pAM320	pUX19 containing the 1.7-kb promoterless *gdhA* gene	This work
pAM321	pBlueScript II KS containing the 1.7-kb promoterless *gdhA* gene	This work
pAM322	pAM321 containing the fusion pr.*nifHc-gdhA*	This work
pAM341	pTR101 with the 1.5-kb fragment containing the *R. etli nifH*c promoter	This work
pAM340	pTR101 containing the *E. coli ghdA* gene under the control of the *R. etli nifH*c promoter; 3.2-kb *KpnI-SacI*	This work

Determination of ureides.

Groups of five plants were decapitated 1 cm above the crown at 18, 25, or 32 dpi. A 3-cm-long rubber tube was attached to collect the xylem sap, and the contents were pooled. Ureides content was determined by the colorimetric assay according to Vogel and Van der Drift (1970).

Light microscopy.

Entire primary roots with fully developed nodules were collected and cut into 100-µm segments with a vibratome (Series 1000, Technical Products International, St. Louis, MO), immediately stained with Epoxy Tissue Stain (Electron Microscopy Sciences FT, Washington, PA), and viewed under a Zeiss optical microscope.

Transmission electron microscopy.

Electron micrographs of nodules sections were obtained as described by Cevallos et al. (1996) and observed with a Zeiss EM-900 transmission electron microscope.

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