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A *gua*B Mutant Strain of *Rhizobium tropici* CIAT899 Pleiotropically Defective in Thermal Tolerance and Symbiosis

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Rhizobium tropici **strain CIAT899 displays a high intrinsic thermal tolerance, and had been used in this work to study the molecular basis of bacterial responses to high temperature. We generated a collection of *R. tropici* CIAT899 mutants affected in thermal tolerance using Tn*5*-*lux*AB mutagenesis and described the characterization of a mutant strain, CIAT899-10T, that fails to grow under conditions of high temperature. Strain CIAT899-10T carries a single transposon insertion in a gene showing a high degree of similarity with the *gua*B gene of *Escherichia coli* and other organisms, encoding the enzyme inosine monophosphate dehydrogenase. The *gua*B strain CIAT899-10T does not require guanine for growth due to an alternative pathway via xanthine dehydrogenase and, phenotypically, in addition to the thermal sensitivity, the mutant is also defective in symbiosis with beans, forming nodules that lack rhizobial content. Guanine and its precursors restore wild-type tolerance to grow at high temperature. Our data show that, in *R. tropici*, the production of guanine via inosine monophosphate dehydrogenase is essential for growth at extreme temperatures and for effective nodulation.**

Additional keywords: guanine biosynthesis, nodule development, prototrophy.

Soil bacteria are often exposed to environmental constraints such as nutrient limitations, variations in pH, and temperature shifts. The ability of rhizobia to persist under high thermal conditions seems to be a prerequisite allowing colonization and subsequently nodule formation (Buttery et al. 1992). Rhizobia establishes symbiosis with legumes by eliciting the formation of nitrogen-fixing root nodules. The symbiosis begins when flavonoids produced by the plant induce the bacteria to synthesize a molecular signal, Nod factor, that stimulates cell divisions in the root, resulting in nodule organogenesis. The bacteria enter the developing nodule via an infection thread, are taken up by plant host cells in an endocyticlike process, and undergo differentiation into a distinct cell type called bacteroid. The culmination of bacterial differentiation is the formation of cells that are capable of fixing atmospheric nitrogen into ammonia, which is assimilated by the plant.

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High soil temperature in tropical areas is indicated to be a major problem for biological nitrogen fixation by common beans (*Phaseolus vulgaris* L.), and variability in thermal tolerance in bean-nodulating rhizobia has been shown (Hungría and Franco 1993; Piha and Munns 1987). Hungría et al. (1993) had examined a collection of isolates from Brazil and identified several bean-nodulating strains able to nodulate beans and fix nitrogen under conditions of high temperature. Michiels et al. (1994) found heat-inducible proteins in heat-sensitive and heat-tolerant strains, and demonstrated acquired thermotolerance in the heat-tolerant strain *Rhizobium tropici* CIAT899. The understanding of how bean-nodulating strains cope with the thermal stress might offer clues useful to improve inoculum strains in a program of bean inoculation (Bolhool et al. 1992; Buttery et al 1992). In this article, we examined a Tn*5*-*lux*AB-induced *R. tropici* CIAT899 mutant (CIAT899-10T) that is affected in its intrinsic thermal tolerance, and showed that strain CIAT899-10T is mutated in the gene encoding for the enzyme inosine monophosphate (IMP) dehydrogenase that participates in the guanine biosynthetic pathway. Strain CIAT899-10T is not a guanine auxotrophic mutant; however, because it was also previously described for other rhizobium auxotrophic mutants in purine (*pur*) genes, CIAT899-10T fails to effectively nodulate bean plants, forming nodules that lack rhizobial content.

RESULTS

Isolation of the thermal-sensitive mutant CIAT899-10T.

Random transposon Tn*5*-*lux*AB mutagenesis was used to generate thermal-sensitive mutants of *R. tropici* CIAT899. A total of 6,000 Tn*5*-*lux*AB–containing strains were tested for growth in minimal medium agar incubated at 40°C. Mutants defective in thermal tolerance were expected to show reduced or no growth at 40°C but wild-type growth at 28°C. Ten mutants that failed to grow at 40°C were examined in Southern hybridization analysis with a Tn*5*-*lux*AB probe, and it was found that seven strains had more than one hybridizing band,

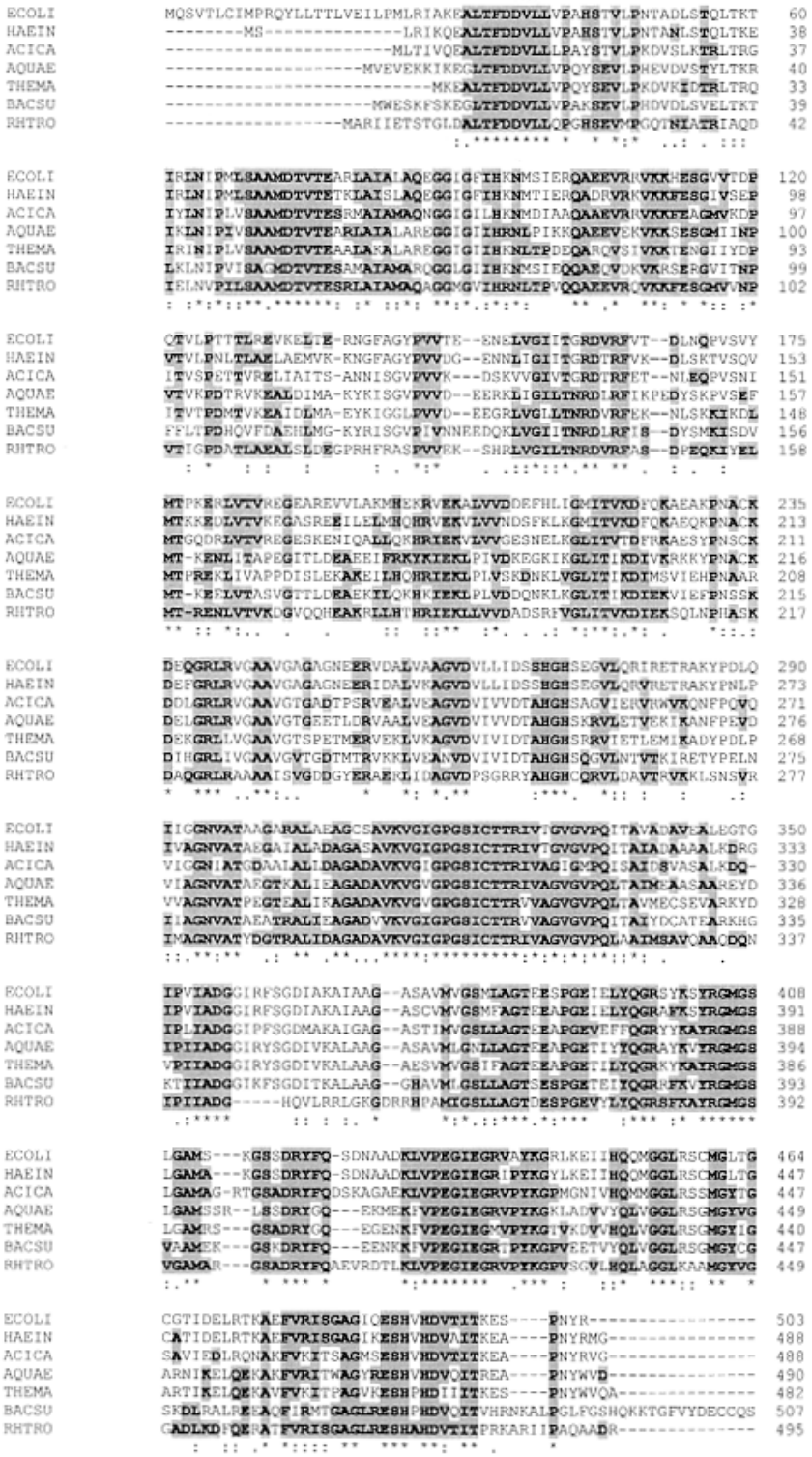

Fig. 1. Multiple alignment analysis of sequences homologous to the GuaB protein. Amino acid sequence similarity of the putative protein product of the transposon tagged open reading frame of strain *Rhizobium tropici* CIAT899-10T (RHTRO) to the GuaB of several organisms. The GenBank/EMBL accession numbers of the sequences given between parenthesis, are as follows: ECOLI (*Escherichia coli*, X02209), HAEIN (*Haemophilus influenzae*, U32708), ACICA (*Acinetobacter calcoaceticus*, X66859), AQUAE (*Aquifex aeolicus*, AE000768), THEMA (*Thermotoga maritima*, AE001789), and BACSU (*Bacillus subtilis*, X55669). Boxes and asterisks denote amino acid residues found identical among all the sequences. Dots indicate conserved positions. Dashes indicate gaps introduced to maximize alignment. Amino acid residue coordinates are given on each line. The five amino acid residues corresponding to the C-terminal end of the GuaB of *B. subtilis* had been omitted.

whereas the other three strains contained a single transposon insertion. The occurrence of more than a single insertion in thermal-sensitive mutants was found to be more frequent than in the case of other phenotypes we were looking for (data not shown). At this time, the reason for this observation is unknown to us. Strain CIAT899-10T, one of the thermal-sensitive mutants showing a single transposon insertion, was further characterized.

The growth rate of strain CIAT899-10T was found to be lower than the parent strain in liquid media at 28°C, with a mean generation time of approximately 4 versus 3 h of wild type (data not shown).

Strain CIAT899-10T is a *gua*B mutant.

To characterize the gene mutated with the Tn*5*-*lux*AB in strain CIAT899-10T, the tagged locus was cloned from the rhizobial genome. This was facilitated by the presence of an origin of replication within the Tn*5*-*lux*AB transposon (Wolk et al. 1991). Total genomic DNA of strain CIAT899-10T was digested with restriction enzyme *Eco*RI, self-ligated, and transferred into *Escherichia coli* via transformation. The DNA sequence of the *R. tropici* fragments adjacent to the transposon DNA was determined by using unique primers corresponding to both ends of the Tn*5*-*lux*AB and also appropriate primers that were deduced of the resulting DNA sequence data. Sequence analysis revealed an open reading frame (ORF) of 1,485 nucleotides showing an ATG start codon preceded by a putative ribosomal binding site (data not shown). The predicted protein deduced of this ORF shares 52% identical residues with the *gua*B gene product of *E. coli* and of several other bacteria (Fig. 1). The *gua*B encodes the enzyme IMP dehydrogenase, which catalyses the conversion of inosine 5′-monophosphate into xanthosine 5′-monophosphate in the guanosine biosynthetic pathway. Extended sequence analysis upstream and downstream from the ORF encoding the *gua*B gene did not reveal homology to the *gua*A gene; therefore, unlike *E. coli*, genes *gua*A and *gua*B are separated in the genome of *R. tropici* (Neuhard and Nygaard 1987).

The activity of IMP dehydrogenase in the extract of *R. tropici* cells was determined to confirm that strain CIAT899-10T is mutated in the structural gene for IMP dehydrogenase. Native polyacrylamide gel electrophoresis (PAGE) of crude extract of wild-type strain revealed by activity staining showed a single protein band. In contrast, no band was observed with the extracts from mutant CIAT899-10T (Fig. 2). Furthermore, it was demonstrated that CIAT899-10T recovered the ability to grow at high temperature when the medium was supplemented with guanine (Fig. 3) and that the mutant was complemented by the plasmid pMC1 carrying the wild-type *gua*B gene. However, this positive effect was not observed when pMC2 (*gua*B gene cloned in opposite orientation) was introduced into strain CIAT899-10T (data not shown). These results clearly demonstrate that the Tn*5*-*lux*AB tagged gene in strain CIAT899-10T is the structural gene for IMP dehydrogenase and that the transposon insertion causes the observed phenotype. It is interesting to note that strain CIAT899-10T did not require guanine to be added for growth on minimal medium at 28°C, indicating that guanine, which is dependent of a functional *gua*B gene product, is required for growth when rhizobia cells are shifted to high temperature. Although high temperature affected growth, cells were found to remain viable under such condition (Fig. 3).

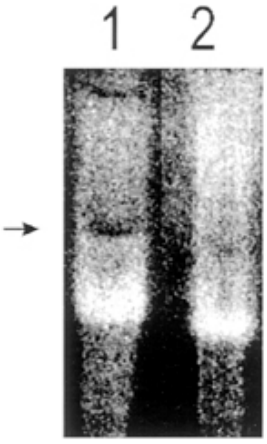

Fig. 2. Native polyacrylamide gel electrophoresis of crude extract of *Rhizobium tropici* stained by inosine monophosphate (IMP) dehydrogenase activity staining. Samples containing 11 µg of protein that were obtained from rhizobia cells grown at 28°C were loaded onto each of the lanes. Electrophoresis and staining were performed according to Miyamoto et al. (1998). Lanes 1 and 2 are wild-type strain CIAT899 and mutant strain CIAT899-10T, respectively. The band corresponding to the IMP-dehydrogenase is indicated with an arrowhead.

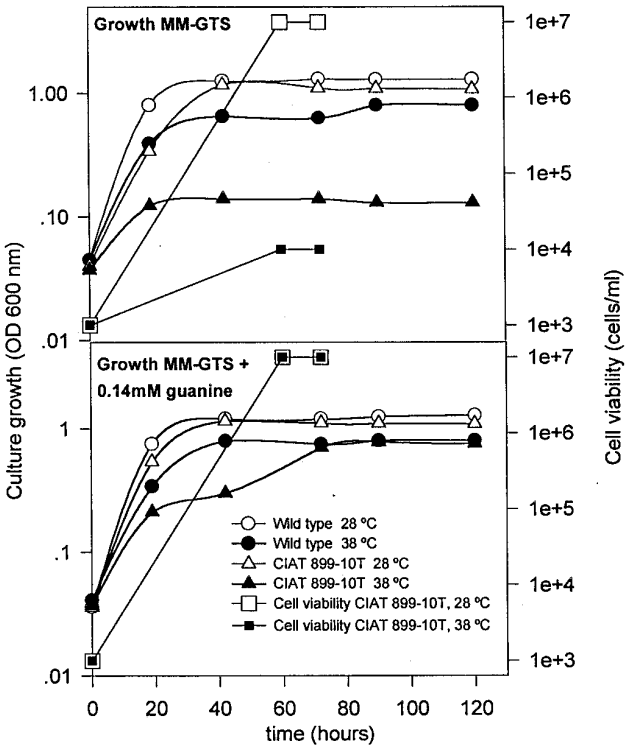

Fig. 3. Recovery of thermal tolerance by *Rhizobium tropici* CIAT899-10T. At time zero, cells of CIAT899 and mutant CIAT899-10T were diluted to optical density at 600 nm (OD_{600}) = 0.08, with minimal medium GTS (MM-GTS, upper panel), and MM-GTS supplemented with 0.14 mM guanine (bottom panel). Growth at 28°C (open symbols) and 38°C (closed symbols), respectively, was monitored by measuring OD_{600}. The data are the means values from three replicate experiments with standard deviation of less than 10%.

***R. tropici* has an alternative pathway for synthesis of guanine.**

The finding that the *gua*B mutant strain CIAT899-10T does not require exogenous guanine for growth at normal temperature suggested that *R. tropici* might have an alternative pathway, bypassing IMP dehydrogenase, as illustrated in Figure 4. In order to test this possibility, growth assays were performed using different supplements. Allopurinol, which is a specific inhibitor of the enzyme xanthine dehydrogenase, prevented growth of mutant strain CIAT899-10T when added to minimal medium. Identical results were obtained if allopurinol was added together with hypoxanthine or adenine, but not when added together with xanthine or guanine (Table 1). This observation suggested the importance of xanthine dehydrogenase in the utilization of guanine precursors. Xanthine dehydrogenase activity was detected in extracts of both the wild-type and the *gua*B mutant strains (data not shown). These results and the observed prototrophy, taken together, demonstrate that, in the absence of IMP dehydrogenase, xanthine serves as precursor instead of inosine monophosphate to yield xanthine monophosphate, and for this, CIAT899-10T relies on the activity of xanthine dehydrogenase. Salvage pathways for purine had been found in bacteria (Neuhard and Nygaard 1987; Stuer-Lauridsen and Nygaard 1998) and a route similar to *R. tropici*, involving xanthine dehydrogenase, was described in *Bacillus subtilis* (Christiansen et al. 1997).

It was also found that addition of guanine precursors, including hypoxanthine and adenine that both require xanthine dehydrogenase activity for their conversion, restored the wild-type ability of strain CIAT899-10T to grow at high temperature (Table 1).

Symbiotic phenotype of strain CIAT899-10T.

In order to examine the symbiotic phenotype of strain CIAT899-10T, we performed plant inoculation experiments on the host legumes common beans and leucaena. Four weeks after inoculation, nodulation was observed in both legumes we assayed. Although the aspect of leucaena plants inoculated with strain CIAT899-10T was healthy and similar to leucaena plants inoculated with the wild type, the aspect of bean plants inoculated with CIAT899-10T was yellow and poor in growth. These observations were in agreement with the results of nitrogenase activity as it was measured by using the acetylene reduction assay (data not shown). The appearance of bean nodules elicited by the mutant strain was very similar to normal nodules, but they were smaller and white (data not shown). Nodules induced by the mutant were numerous, dispersed all over the root, whereas wild-type nodules were mainly located in the upper part of the root next to the crown. Microscopic observation revealed clear differences between wild-type and mutant nodules. Wild-type–induced nodules exhibited infection threads and a central zone with rhizobia-infected cells, whereas, in the case of mutant nodules, few infection threads

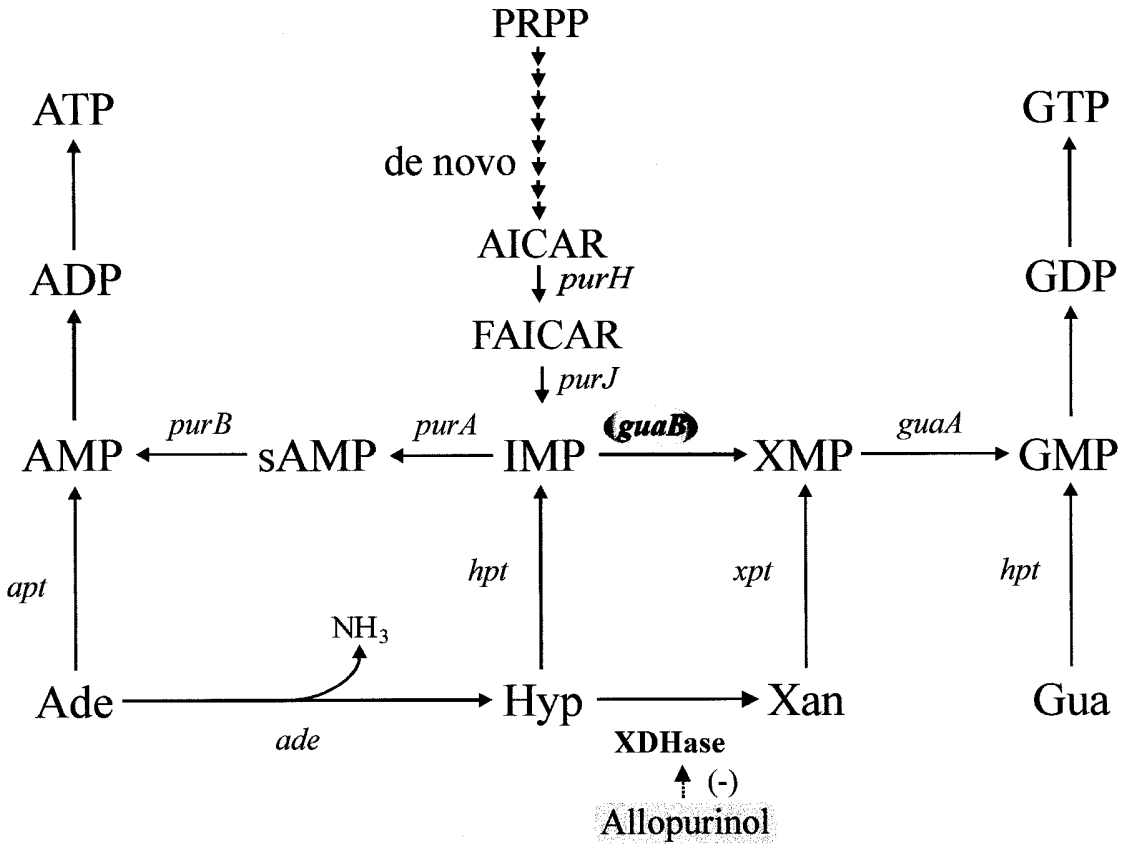

Fig. 4. De novo synthesis of ATP and GTP in *Rhizobium tropici*. The alternative route via xanthine dehydrogenase (XDHase) for guanine biosynthesis and its specific inhibitor allopurinol are indicated. The gene *gua*B mutated in strain CIAT899-10T involved in the conversion of inosine monophosphate (IMP) into xanthosine monophosphate (XMP) is boxed. The other reactions are identified by their gene symbols.

were observed and release of rhizobia within the cells was not detected (Fig. 5). Bacteria were clearly observed in the intercellular space. Cells were vacuolated with large starch grains. In contrast, strain CIAT899-10T carrying plasmid pMC1 was found to form wild-type nodules, which demonstrated that the wild-type *gua*B sequence complemented both defective phenotypes we had observed (data not shown).

These results are consistent with the Ndv⁻ phenotype described previously for *pur* mutants of *R. etli* and other rhizobia (Djordjevic et al. 1996; Newman et al. 1994, 1995; Vandenbosch et al. 1985).

Investigation of heat shock proteins and accumulation of guanosine tetraphosphate (ppGpp).

In order to gain insight into the molecular basis of the thermal-sensitive phenotype of strain CIAT899-10T, we investigated the pattern of expression of signal molecules that are known to be involved in stress response. In *E. coli*, protein degradation and chaperone activation play roles in communicating environmental stress-to-stress response transcriptional factors (Gamer et al. 1996). We investigated the induction of heat shock proteins (HSPs) in the strain CIAT899-10T as follows. Rhizobial cells were incubated in the presence of ^{35}S-methionine and samples were removed before and 1 h after they shifted from 28 to 42°C. Samples were subjected to sodium dodecyl sulfate-PAGE and proteins revealed by autoradiography (Michiels et al. 1994; Narberhaus et al. 1996). It was found that the wild-type strain and strain CIAT899-10T have the same pattern of HSPs (data not shown). Therefore, no obvious correlation between induction of HSP and tolerance to high temperatures was found in the mutant strain CIAT899-10T.

It is known that levels of ppGpp increase in bacteria under conditions such as carbon or nitrogen starvation, oxidative stress, and temperature stress (Ault-Riché et al. 1998; Gallant et al. 1977; Gentry et al. 1993; Pao and Gallant 1979; Van-Bogelen et al. 1987). Assuming that ppGpp communicates stress signals to the thermal stress response genes and that, in the *gua*B mutant, the availability of GMP for ppGpp synthesis could be limited, we quantified levels of ppGpp in the wild-type strain and mutant strain CIAT899-10T. ppGpp was determined by high-performance liquid chromatography after formic acid extraction (Howorth and England 1999). In our studies, we found that the wild-type strain and mutant do not accumulate ppGpp following thermal shock at 50°C, indicating that ppGpp is not required in order to initiate responses that permit *R. tropici* to grow at high temperature. Howorth and England (1999) had described *R. tropici* CIAT899 to behave as a relaxed strain.

Table 1. Effect of allopurinol and guanine precursors on growth of wild-type and *gua*B mutant strains

Strain, supplementation[a]	Growth at[b] 28°C	38°C
Wild type CIAT899		
None	+	+
Allopurinol	+	+
Mutant CIAT899-10T		
None	+	−
Allopurinol	−	−
Hypoxanthine	+	+
Allopurinol plus hypoxanthine	−	−
Xanthine	+	+
Allopurinol plus xanthine	+	+
Adenine	+	+
Allopurinol plus adenine	−	−

[a] Allopurinol was added at a final concentration of 1 mM guanine, and the other nucleotides were added at a final concentration of 0.14 mM.
[b] Growth of wild-type and *gua*B mutant strains in liquid minimal medium GTS supplemented with guanine and precursors, monitored by optical density at 600 nm.

DISCUSSION

Strain *R. tropici* CIAT899-10T was generated by the insertion of a single copy of transposon Tn*5-lux*AB into strain *R. tropici* CIAT899. In this article, we present the results of molecular and biochemical studies showing that the locus mutated in CIAT899-10T contains the gene *gua*B encoding the enzyme inosine 5-monophosphate dehydrogenase. The alteration of the *R. tropici gua*B gene resulted phenotypically pleiotropic, affecting both the thermal tolerance and the symbiosis with beans. In rhizobia, more data had been published regarding the symbiotic properties of purine auxotrophs; therefore, hereafter, we will first address our discussion to the symbiosis phenotype of CIAT899-10T, and later to the issue of thermal tolerance.

Previous reports have shown that rhizobial purine auxotrophs are defective in symbiosis with alfalfa, bean, pea, siratro, and soybean (Djordjevic et al. 1996; Kim et al. 1988; Newman et al. 1994, 1995; Scherrer and Denaire 1971; Vandenbosch et al. 1985). These mutants behave as auxotrophs, requiring purine precursors for growth on laboratory minimal medium. The mutations had been mapped in several *pur* genes that specify early steps of the purine biosynthetic pathway before the production of the precursor 5-amino-4-carboxamide ribonucleotide (AICAR). It was shown that *pur* auxotroph mutants induce pseudonodules in several legumes and that addition of AICAR to plant medium promote infection of bean, soybean, pea, and siratro (Djordjevic et al. 1996; Newman et al. 1995; Niner and Hirsch 1998). These results, which were obtained with mutants in genes corresponding to early steps of the purine biosynthetic pathway, led the authors to conclude that AICAR produced by rhizobia is required for infection. However, our results demonstrated that strain CIAT899-10T, a mutant in the *gua*B and therefore blocked after AICAR, also formed structures superficially resembling nodules; however, because nodule development was found incomplete, the *gua*B mutant is also among those designated as Ndv. We believe that this is the first report in *Rhizobium* spp. in which the isolation and characterization of a *gua*B mutant strain, affected in a step close to the end of the guanine biosynthetic pathway, are described. The experimental approach we used for the isolation of the *gua*B mutant was based in selecting for thermal sensitivity, otherwise due to the xanthine dehydrogenase activity of rhizobia, the chance to isolate guanine auxotroph mutants should be rather low. Nevertheless, our results indicated that a compound of the guanine biosynthetic pathway other than the precursor AICAR is important for successful infection and that the proposed significance of AICAR for symbiosis should be revised.

The genetics and biochemistry of guanine biosynthesis had been extensively studied in *E. coli*. The genes *gua*A and *gua*B of *E. coli* together form an operon (Mehra and Drabble 1981;

Neuhard and Nygaard 1987). Analysis of the DNA region flanking the *R. tropici gua*B gene did not reveal homology to the *gua*A gene; therefore, it seems that the genetic organization may differ from that found in *E. coli*. We found that supplementation with guanine and also with precursors for the production of xanthine via xanthine dehydrogenase restored the wild-type ability of mutant strain CIAT899-10T to grow under conditions of high temperature. Therefore, it seems unlikely that the observed inability of strain CIAT899-10T to grow at high temperature may result from a putative thermal sensitivity of xanthine dehydrogenase.

In *E. coli*, the major flux through the guanylate pool during balanced growth is directed into nucleic acid biosynthesis. We assume that, in the mutant strain CIAT899-10T, requirements

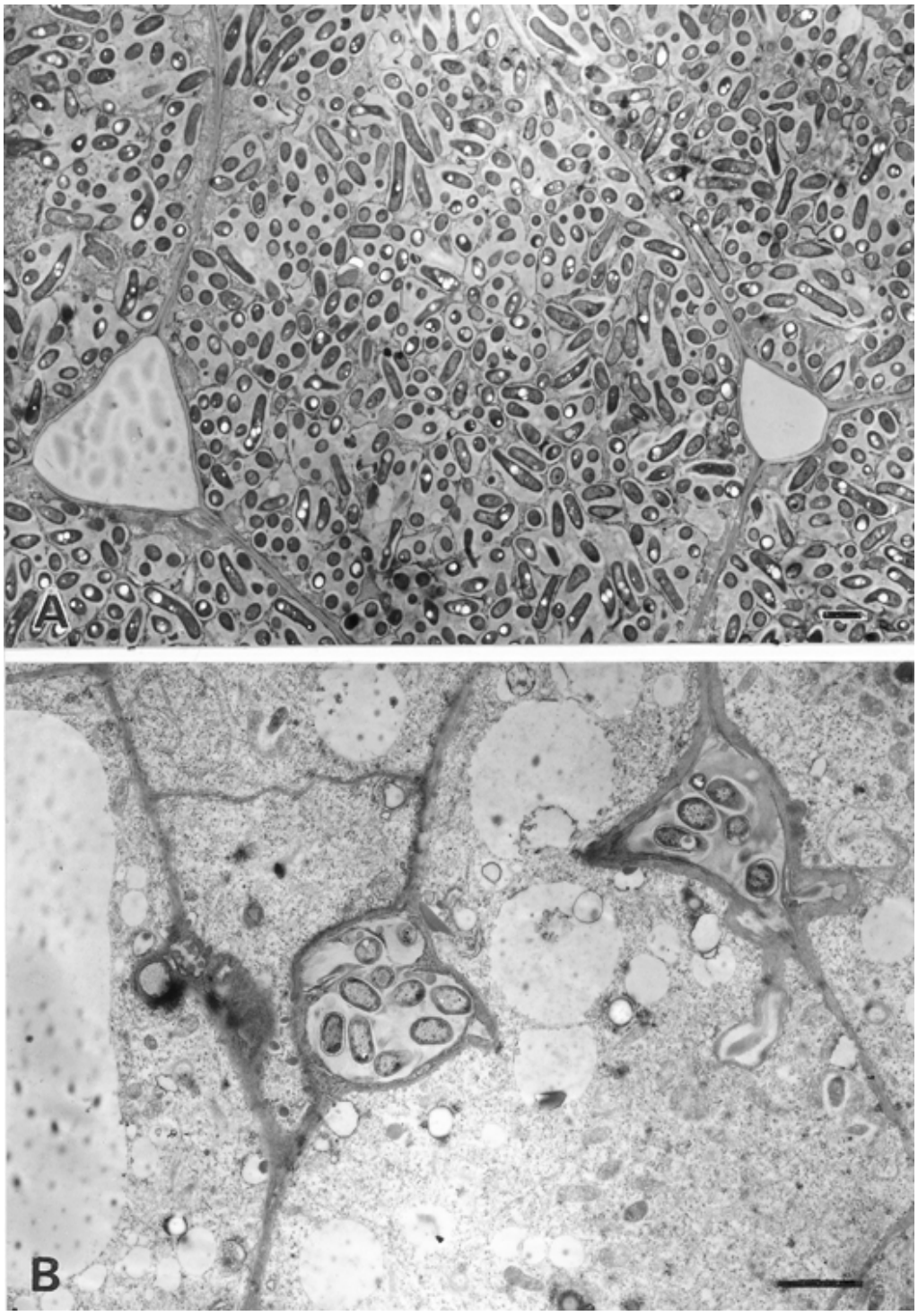

Fig. 5. Electron microscopic examination of bean nodules induced by wild-type strain CIAT899 and mutant strain CIAT899-10T. **A,** Clear presence of rhizobia in cells of wild-type nodules. **B,** Empty nodules induced by the mutant strain, in which bacteria are found present in the intercellular space. Bar = 2 μM.

of guanylates for vegetative growth is fulfilled by the alternative biosynthetic pathway via xanthine dehydrogenase; however, it does not fulfill requirements that appear under special conditions such as high temperature. Supplementation with guanine and precursors reverses to thermal tolerance.

How guanylates can play a role in determining tolerance to changes in environmental temperature remains an interesting yet still intriguing issue. The guanine biosynthesis pathway also provides precursors for several molecules considered to play roles as physiological signals. For instance, ppGpp, which is produced via the *rel*A gene product, participates as messenger in the stringent response and other stressing conditions (Ault-Riché et al. 1998; Belitsky and Kari 1982; Gentry et al. 1993; Howorth and England 1999; Kleiner and Phillips 1981; Van Bogelen et al. 1987). However, our data provided no indication that accumulation of ppGpp is required to initiate response to thermal stress. It had been described that GTP-binding proteins are involved in signal transduction in prokaryotes and eukaryotes. The GTP-binding protein Era is essential for *E. coli* growth, and its depletion from cells causes pleiotropic effect (Lerner and Inouye 1991). Furthermore, it had been demonstrated that a decrease in the GTP pool size is associated with the morphological differentiation of *Bacillus subtilis* and *Streptomyces* spp. (Lopez et al. 1981; Okamoto et al. 1997; Okamoto and Ochi 1998; Scott and Haldenwang 1999). In these organisms, the GTP-binding protein Obg senses low intracellular levels of GTP and eventually triggers initiation of the differentiation. Conditions in which synthesis of guanine is negatively affected, such as guanine requiring auxotrophic mutants or by the addition of inhibitors of GMP synthesis, induce differentiation in rich media in which cells normally do not sporulate. These results provide evidence that changes in the size in the guanylate pool are important to determine cellular changes and, therefore, gene activation. Our observations indicated that infection by strain CIAT899-10T is blocked before the bacterial release within the nodule cells, and therein, after, the bacteria differentiate into bacteroids, a cellular process that had been compared with sporulation. Although it can be speculated that GTP-binding proteins that sense size of the guanylate pool may be involved in the thermal tolerance and in the late steps of infection, this possibility remains to be demonstrated. Nevertheless, these data highlight the possible diverse targets for guanylates to have an impact on cellular physiology. Our results add further evidence of the importance of metabolic production of guanine precursors for nodule development and add a novel requirement for guanine in the thermal tolerance by *Rhizobium* spp.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media.

R. tropici CIAT899 (streptomycin resistant, Sm^r), a bean-nodulating strain described by Martínez-Romero et al. (1991), was grown at 28°C in tryptone-yeast extract (TY) medium (Beringer 1974) or minimal GTS medium (Kiss et al. 1979). Strain *E. coli* DH5α (Hanahan 1983) derivatives harboring the Tn*5*-*lux*AB–containing plasmid pRL1063a (Wolk et al. 1991) or the helper plasmid pRK2013 (Ditta et al. 1980) were grown at 37°C in Luria broth (LB) supplemented with kanamycin (LB-Km) at 50 μg per ml.

Random transposon mutagenesis.

Transposon Tn*5*-*luxAB* mutagenesis of *R. tropici* CIAT899 was carried out using the protocol for Tn*5* mutagenesis of *Rhizobium* spp. described by de Bruijn and Rossbach (1994). Cells of the donor strain *E. coli* DH5α harboring the suicide plasmid pRL1063a or the helper plasmid pRK2013, and the recipient *R. tropici* CIAT899 strain, were grown in LB-Km and TY, respectively, washed with fresh TY medium, and concentrated 10-fold in TY medium. Equal amounts (100 μl) of donor, helper, and recipient cells were mixed and spotted on TY plates. After 24 h of incubation at 28°C, the mating mixtures were suspended in sterile distilled water and plated on selective TY medium, supplemented with Sm (400 μg per ml) and neomycin (Nm; 100 μg per ml).

Screen of temperature-sensitive mutants.

R. tropici strains carrying Tn*5*-*lux*AB insertions were screened for temperature sensitivity by using toothpicks to streak individual colonies onto GTS plates that were incubated at 28 and 40°C, respectively. Transconjugants that grew poorly or not at all at 40°C, but grew well at 28°C, were selected for further analysis.

Growth and stress survival tests.

Starter cultures were generated in GTS liquid medium, supplemented with antibiotics, and allowed to grown to mid-log phase. Duplicate flasks, containing 50 ml of GTS medium, were inoculated with an aliquot of starter culture to yield an initial optical density at 600 nm of 0.06 (equivalent to approximately 10^4 cells per ml). The cultures were incubated under aeration at 28 or 38°C and growth was periodically monitored by measuring optical density (at 600 nm) and by determining the viable cells on TY medium. The experiments were replicated at least twice.

IMP dehydrogenase activity.

IMP dehydrogenase activity was determined by electrophoresis and activity staining, essentially as described by Miyamoto et al. (1998), except that samples were concentrated by ultrafiltration on ultrafuge cartridge 100,000 NMWC (MSI, Westboro, MA, U.S.A.) instead of dialysis. The protein concentration of the samples was determined by the bicinchoninic acid assay (Sigma-Aldrich, St. Louis, U.S.A.).

DNA isolation and manipulations.

Plasmid DNA was prepared by the alkaline method as described by Kragelund et al. (1995). Total genomic *R. tropici* DNA isolation, restriction enzyme digestions, ligations, and Southern blotting experiments were carried out according to procedures previously described (Aguilar and Grasso 1991; Sambrook et al. 1989). Construction of plasmid pMC1 carrying the *R. tropici* CIAT899 wild-type *gua*B coding sequence was done as it follows. A 1,578-base pair (bp) DNA fragment from *R. tropici* CIAT899 encompassing the ORF of the *gua*B gene and the upstream adjacent ribosome binding sequence was obtained by polymerase chain reaction (PCR) amplification with a pair of oligonucleotide primers that were deduced of the *gua*B DNA sequence data (forward primer 5′-CGTAG-ACGAATTCGTTCCGGG-3′ and reverse primer 5′-GATTT-AAGGAATTCTGCAGTA-3′). The PCR DNA fragment was purified and cloned into the vector plasmid pGEM (Promega

Corp., Madison, WI, U.S.A.). The 1,578-bp fragment was excised as an *Eco*RI fragment and cloned into the *Eco*RI recognition site present in the gene encoding cloramphenicol resistance (*cat*) of vector plasmid pSUP204. The new plasmids, containing the *gua*B coding sequence downstream from the *cat* gene, were designated pMC1 (correct orientation with respect to the *cat* promoter) and pMC2 (opposite orientation).

DNA sequence analysis.

Sequencing of double-stranded plasmid DNA was performed using the dideoxy method of Sanger, using Sequenase kits (US Biochemicals, Cleveland, OH, U.S.A.). To determine the *R. tropici* DNA sequence on both sides of the transposon insertion, the procedure described by Millcamps et al. (1998) was used. DNA sequence data were analyzed using the Wisconsin Package version 9.0 GCG (Madison, WI, U.S.A.) program. Similarities were examined with the BLAST program (Altschul et al. 1990). The accession number is AF272827.

Nodulation assays.

R. tropici strains were assessed for their symbiotic phenotype by inoculation on seedling roots of bean and leucaena, respectively. *Leucaena leucocephala* seed was first scarred by heat treatment (80°C, 10 min). Seed were sterilized by soaking for 3 min in 95% (vol/vol) ethanol, followed by 15 min in sodium hypochloride (8.25 g liter^{-1}), and then rinsed thoroughly with sterile distilled water. Sterilized seed were placed on top of sterile agar and water and incubated in darkness at 28°C.

The inoculation with rhizobial suspensions was performed on roots of 5-day-old seedlings. Seedlings were transferred into pots containing sterile vermiculite and incubated in a glasshouse with a temperature range of 25 ± 5°C. At 3 to 4 weeks after inoculation, the plants were examined for the presence or absence of root nodules. Whole plant nitrogen fixing activity was determined by acetylene reduction assay.

Microscopy.

Light and electron microscopy were performed according to the procedure described by Aguilar et al. (1985).

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