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Nodulating ability of *Rhizobium tropici* is conditioned by a plasmid-encoded citrate synthase

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Summary

***Rhizobium* species elicit the formation of nitrogen-fixing root nodules through a complex interaction between bacteria and plants. Various bacterial genes involved in the nodulation and nitrogen-fixation processes have been described and most have been localized on the symbiotic plasmids (pSym). We have found a gene encoding citrate synthase on the pSym plasmid of *Rhizobium tropici*, a species that forms nitrogen-fixing nodules on the roots of beans (*Phaseolus vulgaris*) and trees (*Leucaena* spp.). Citrate synthase is a key metabolic enzyme that incorporates carbon into the tricarboxylic acid cycle by catalysing the condensation of acetyl-CoA and oxaloacetic acid to form citrate. *R. tropici pcsA* (the plasmid citrate synthase gene) is closely related to the corresponding genes of Proteobacteria. *pcsA* inactivation by a Tn*5–mob* insertion causes the bacteria to form fewer nodules (30–50% of the original strain) and to have a decreased citrate synthase activity in minimal medium with sucrose. A clone carrying the *pcsA* gene complemented all the phenotypic alterations of the *pcsA* mutant, and conferred *Rhizobium leguminosarum* bv. *phaseoli* (which naturally lacks a plasmid citrate synthase gene) a higher nodulation and growth capacity in correlation with a higher citrate synthase activity. We have also found that *pcsA* gene expression is sensitive to iron availability, suggesting a possible role of *pcsA* in iron uptake.**

Introduction

The discovery of the tricarboxylic acid (TCA) cycle and its central role in carbon metabolism was one of the most significant events in the development of modern biochemistry. Citrate synthase is considered to be the rate limiting step in the cycle. With a few exceptions, the TCA cycle has been found in the majority of organisms (Weitzman and Danson, 1976).

Up until now, genes encoding central metabolic pathway enzymes, such as the TCA genes, have been located on chromosomes in bacteria, while genes considered accessory or dispensable have been located on plasmids. In *Rhizobium*, genes involved in plant interaction have been located on the so-called symbiotic plasmids (pSym). These carry nodulation and nitrogen-fixation genes that determine the formation of symbiotically effective nodules on the roots of specific legumes. We have described a new species of *Rhizobium* (*Rhizobium tropici*) that effectively nodulates *Phaseolus vulgaris* and *Leucaena* spp., and is tolerant to high temperatures and acidity (Martinez-Romero *et al.*, 1991). In this paper we report the sequence of a functional citrate synthase encoded by the *R. tropici* symbiotic plasmid, some features of its transcriptional regulation, and its role in nodulation.

Results and Discussion

R. tropici CFN299 was mutagenized with Tn*5–mob* (Simon, 1984); Tn*5–mob* insertions on the 400 kb symbiotic plasmid were selected as follows. Five-thousand mutants were individually mated (Martínez *et al.*, 1987) with a plasmid-free *Agrobacterium tumefaciens* strain GMI9023 (Rosenberg and Hughet, 1984). *Agrobacterium* transconjugants bearing the Sym plasmid were identified by positive hybridization to the *R. phaseoli nifH* gene (Quinto *et al.*, 1985). The corresponding parental *Rhizobium* strains (300 mutants), with Tn*5–mob* insertions on pSym, were selected and tested for nodulation kinetics and acetylene reduction activity in duplicated assays with *P. vulgaris* bean plants (Martinez *et al.*, 1985; Martínez-Romero and Rosenblueth, 1990). A mutant (CFNE130) with a nodulation-deficient phenotype, but a normal nitrogen-fixing capacity, was chosen for further analysis. Its Tn*5–mob* single insertion and the flanking regions were cloned in pUC18. The DNA sequence revealed that the Tn*5–mob* was inserted into an open reading frame (ORF) that resembles prokaryotic citrate synthase genes (Fig. 1). Two experiments were performed to verify further the location of this sequence on the plasmid: (i) a *pcsA* internal fragment (Fig. 1A) was used as

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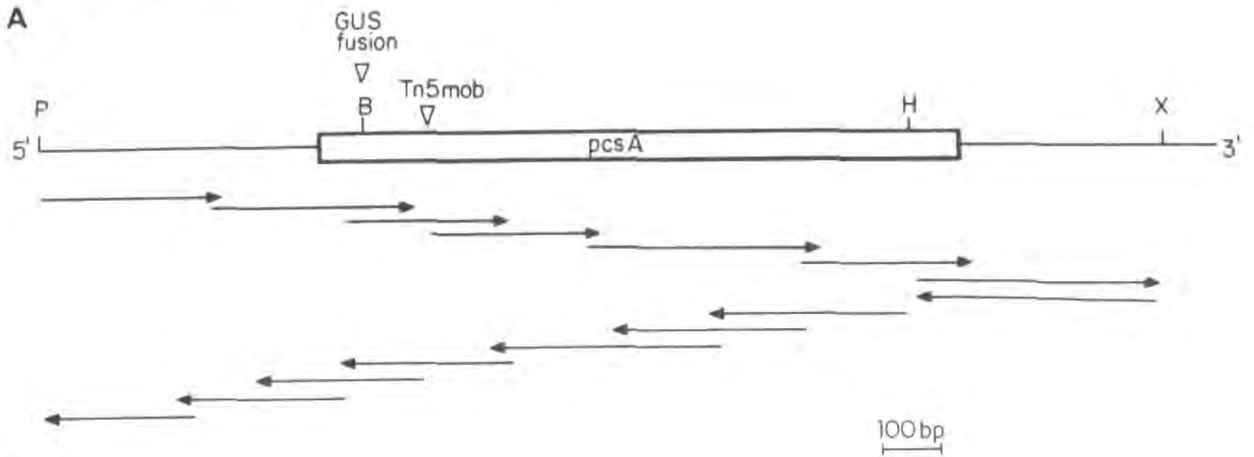

B

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     CTGCAGATCGGCTCGACCCCGAACTTTGAGCGGTGTTCGTCGATGAAGGAAATCATCGCTTCAGTGGGCGGTCGAGCTCCGCTGGGCAAAATAAGCCGACGCCTTACGCA   110
     AAATCTCATTGGCCTGTCGAAGCTCGCGGTTCTCCCGCTCAAGGGCCTTCATCTTCTCGGCGACATCGCTTGGAAGGCCTGCTCGTTTGCCGCTGTCAACATCGGCTTTC   220
     TTCACCCACTCATGCAGCGTATGTGCCGAGCAGCCAATCTTGGCGCTATGGATGAAACTGCCGCCCACCGGATGGATGCTCGGCCTCATGGTCCAAAACCATGCGGATGG   330
     CTCGGTCACGAACTTCAGGTGAAATTTGTTGGTCGTCTTGCTCATATCGGCTCCACTTTCTCACGAGTTGGAGCCTCCGCGAAACCCGGCGCGGTTCACCCCCTCTGCGG   440
     GAAGACAATTAGAACACAGATCAGTTCATTCGGCAGCTGTTAGTTCCCAGCTAGCGAGCCCTGAGGAAAAATATCATGGATAACAACAATGCTTGTGTCTTAGTCGACGG   550
                                                                                        MetAspAsnAsnAsnAlaCysValLeuValAspGl
     CCATAGTGCCGAATTGAAACTTCGATCAAGCACGATCGGTCCGAACGTCCTCGGCATTGGATCCCTCTACGAGCAGACGAAGATGTTCACCTATGATCCTGGCTTCACTT   660
 12  yHisSerAlaGluLeuLysLeuArgSerSerThrIleGlyProAsnValLeuGlyIleGlySerLeuTyrGluGlnThrLysMetPheThrTyrAspProGlyPheThrS
     CGACTGCGTCGTGCGAGTCCAGCATCACCTTTATTGATGGCGACGAAGGCGTTCTGCTGCATCGCGGTTATCCGATCGAACAGCTTGCCGAGCACGGCGACTTCCTCGAA   770
 49  erThrAlaSerCysGluSerSerIleThrPheIleAspGlyAspGluGlyValLeuLeuHisArgGlyTyrProIleGluGlnLeuAlaGluHisGlyAspPheLeuGlu
     GTCTGTTACCTGCTGCTCTACGGCGAATTGCCGACCGCAGCCCAGAAGAAGGACTTCGACTATCGGGTCGTGCACCACACCATGGTGCATGAACAAATGTCCCGCTTCTT   880
 86  ValCysTyrLeuLeuLeuTyrGlyGluLeuProThrAlaAlaGlnLysLysAspPheAspTyrArgValValHisHisThrMetValHisGluGlnMetSerArgPhePh
     CACCGGTTTCCGCCGCGATGCGCATCCGATGGCCGTCATGTGCGGCTGCGTCGGCGCTCTGTCGGCCTTCTATCACGACTCCACCGACATCACCGATCCGCACCAGCGCA   990
122  eThrGlyPheArgArgAspAlaHisProMetAlaValMetCysGlyCysValGlyAlaLeuSerAlaPheTyrHisAspSerThrAspIleThrAspProHisGlnArgM
     TGGTCGCAAGCCTTCGTATGATCGCCAAGATGCCGACGCTTGCCGCCATGGCCTACAAGTACCATATCGGCAGCCCTTCGTTTACCCGAAGAACGATCTCGACTATGCG  1100
159  etValAlaSerLeuArgMetIleAlaLysMetProThrLeuAlaAlaMetAlaTyrLysTyrHisIleGlyGlnProPheValTyrProLysAsnAspLeuAspTyrAla
     TCGAATTTCCTGCGCATGTGCTTTGCCGTGCCCTGCGAGGAATATGTGGTCAATCCGGTGCTTGCCCGCGCATGGACCGCATCTTCATCCTGCACGCGGATCATGAACA  1210
196  SerAsnPheLeuArgMetCysPheAlaValProCysGluGluTyrValValAsnProValLeuAlaArgAlaMetAspArgIlePheIleLeuHisAlaAspHisGluGl
     GAACGCATCGACCTCGACGGTTCGCCTCGCCGGCTCTTCCGGCGCCAATCCCTTTGCTTGCATCGCCGCCGCATCGCATGCCTCTGGGGCCCGGCCCATGGCGGCGCCA  1320
232  nAsnAlaSerThrSerThrValArgLeuAlaGlySerSerGlyAlaAsnProPheAlaCysIleAlaAlaGlyIleAlaCysLeuTrpGlyProAlaHisGlyGlyAlaA
     ACGAACGTGCGCTCAACATGCTGACGGAAATCGGCACGGTCGACCGCATTCCGGAATATATCGCCCGCGCAAGGACAAGAACGATCCGTTCCGATTGATGGGCTTCGGT  1430
269  snGluArgAlaLeuAsnMetLeuThrGluIleGlyThrValAspArgIleProGluTyrIleAlaArgAlaLysAspLysAsnAspProPheArgLeuMetGlyPheGly
     CATCGCGTTTACAAGAACTACGATCCGCGCGCCAAGATCATGCAGAAGACGGCGCACGAGGTCCTCGGCGACTCGGCATCAAGGACGATCCGCTGCTCGACATCGCGAT  1540
306  HisArgValTyrLysAsnTyrAspProArgAlaLysIleMetGlnLysThrAlaHisGluValLeuGlyGluLeuGlyIleLysAspAspProLeuLeuAspIleAlaIl
     CGAACTGGAGCGTATCGCGCTGACCGATGACTATTTCATCGAGAAGAAGCTTTACCCGAATGTCGACTTCATTCCGGCATCACGCTGAAGGCGCTCGGCTTCCCCACGA  1650
342  eGluLeuGluArgIleAlaLeuThrAspAspTyrPheIleGluLysLysLeuTyrProAsnValAspPheTyrSerGlyIleThrLeuLysAlaLeuGlyPheProThrT
     CCATGTTCACGGTACTATTTGCGCTCGCCCGCACCGTCGGCTGGATTGCCCAGTGGAACGAGATGATCGAGATCCGGATCAGCGTATCGGCCGCCCGCGCCAGCTCTAT  1760
379  hrMetPheThrValLeuPheAlaLeuAlaArgThrValGlyTrpIleAlaGlnTrpAsnGluMetIleGluAspProAspGlnArgIleGlyArgProArgGlnLeuTyr
     ACCGGCGCACCGCTGCGCGAATACGTTCCGCTTTCAAAGCGCTGAGCGACATCCGTGATGAAACGAATACCGGCTGCTCAGCTGCGATCTCGCAGGGTTGAATGCAATG  1870
416  ThrGlyAlaProLeuArgGluTyrValProLeuSerLysArgEnd
     GCGACCGAACAGTGGTGCTTGACGAATCTTGCTTCCCTTGCGAAGGCCTAGCGCGAGCATGCGAAGACGTGAAAGCGACCGTTCTGCTCGTTGAACTCAGCGACTTCGC  1980
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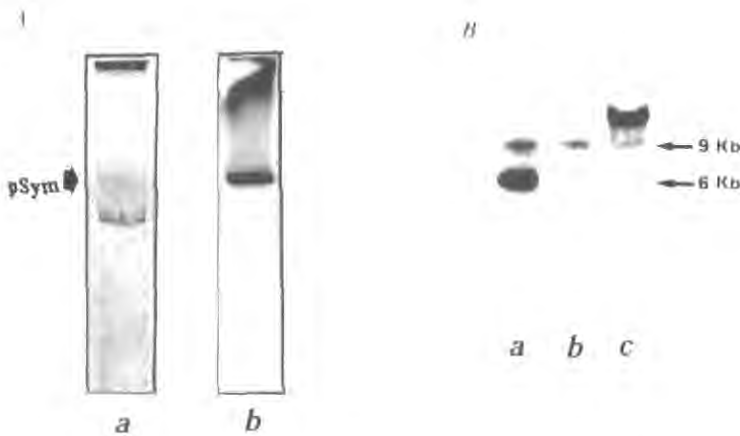

Fig. 2. A. (a) Plasmid profile of strain CFN299. (b) Autoradiogram of the same plasmid profile after hybridization with the *pcsA Bam*HI–*Hin*dIII internal fragment.
B. Autoradiogram of Southern blot *Eco*RI-digested genomic DNAs hybridized with the same *Bam*HI–*Hin*dIII *pcsA* internal fragment. The 6 kb hybridizing band corresponds to the plasmid citrate synthase and the 9 kb band corresponds to the chromosomal citrate synthase gene. (a) Wild-type strain CFN299; (b) CFNE299-10, a pSym spontaneous deletion mutant and (c) CFNE130, a *pcsA*–Tn*5* insertion mutant.

a probe for hybridization against a CFN299 plasmid profile: a positive hybridization was found in pSym (Fig. 2A). Southern blot hybridization of strains CFN299, CFNE130 and CFNE299-10 (a spontaneous symbiotic plasmid deletion mutant) showed that CFN299 has two hybridization bands, one corresponding to the plasmid-located (6 kb) and the other to the chromosomal gene (9 kb). CFNE299-10 showed only the 9 kb band, while CFNE130 has the expected altered hybridization pattern owing to the Tn*5–mob* insertion (Fig. 2B). (ii) A symbiotic plasmid with a Tn*5* derivative, Tn*5-233* (De Vos *et al.*, 1986), located in a site other than the *pcsA* gene, was transferred to a CFNE130 rifampicin-resistant strain (CFNE131). The transconjugant strain CFNE132 loses the original *pcsA* mutant plasmid, as revealed by hybridization with a *pcsA* internal fragment (data not shown). These data show unambiguously a symbiotic plasmid location of the *pcsA* gene.

The *pcsA* deduced amino acid sequence showed 66, 67, 62 and 67% homology with the products of the *gltA* gene of *Escherichia coli* (Ner *et al.*, 1983), *Pseudomonas aeruginosa* (Donald *et al.*, 1989), *Rickettsia prowazekii* (Wood *et al.*, 1987) and *Acetobacter aceti* (Fukaya *et al.*, 1990), respectively. The cladogram, which considers the genetic distance between several citrate synthase genes, clearly locates the *pcsA* gene among those from Gram-negative bacteria (Fig. 3). The conserved amino acids of citrate synthase, constituting the active site involved in substrate binding (Bhayana and Duckworth, 1984; Weigand *et al.*, 1984), are also found in the *pcsA*-deduced protein (Fig. 1B). In *E. coli*, *sdhCDAB* and *sucABCD*, which code for TCA cycle enzymes, are located upstream of *gltA* (Nimmo, 1987). In contrast the 2.5 kb sequence preceding *pcsA* does not have any homology to other TCA cycle genes (data not shown).

CFNE130 grows less and has around 40% of the wild-type citrate synthase activity when grown in minimal media (MM) with sucrose as a carbon source (Fig. 4). Sucrose is the main carbon compound in *P. vulgaris* bean phloem (Fisher, 1978) and may be an important nutrient for the bacteria in the infection thread. The reduced nodulation capacity (Fig. 5) may be related to growth deficiencies of CFNE130 during the infection process. No growth-rate differences or decreased citrate synthase activity were observed when bacteria were grown in a complete medium (data not shown).

The wild-type *pcsA* gene and its flanking regions were cloned in the broad-host-range pRK7813 plasmid (see the *Experimental procedures*) and introduced into the CFNE130 mutant. This clone restored the phenotypic alterations of the mutant strain, namely nodulation ability

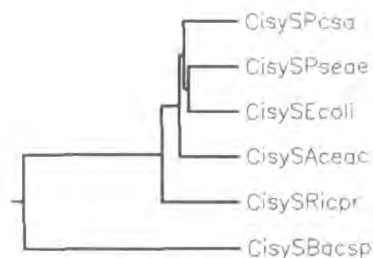

Fig. 3. Relationship between the complete *R. tropici* plasmid-encoded citrate synthase pcsA gene and other citrate synthase (*gltA*) genes. CisySPcsa, *R. tropici pcsA* gene; CisySPseae, from *P. aeruginosa*; CisySEcoli, from *E. coli*; CisySAceac, from *A. aceti*; CisySRicpr, from *R. prowazekii*; CisySBacsp, from *Bacillus subtilis*.

Fig. 1. A. Physical map of the plasmid citrate synthase (*pcsA*) locus showing the GUS and Tn*5-mob* insertions. The arrows depict the extent and direction of the nucleotide sequencing procedure.
B. Complete nucleotide sequence and deduced amino acids of the *pcsA* gene. Amino acids proposed to be involved in substrate binding are marked with an & symbol. Identity between *E. coli* citrate synthase and the deduced *pcsA* gene product is indicated by dots; the & marked residues are also conserved. Restriction sites: P, *Pst*I; B, *Bam*HI; H, *Hin*dIII; and X, *Xba*I.

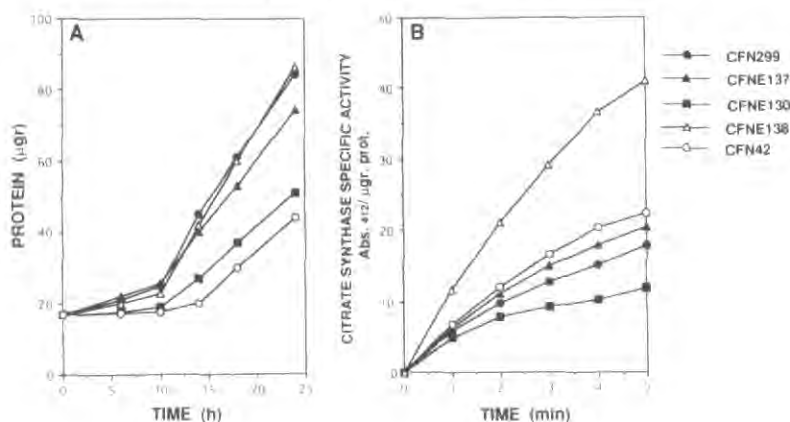

Fig. 4. Growth rate (A) and (B) citrate synthase activity. Bacteria were grown in MM supplemented with 0.2% sucrose as carbon source. CFN299, *R. tropici* wild-type strain; CFNE130, *pcsA* mutant; CFNE137, a *CFNE130 transconjugant that habours a cloned pcsA* gene; CFN42, a *R. leguminosarum* bv. *phaseoli* wild-type strain; CFNE138, a CFN42 transconjugant harbouring a cloned *pcsA* gene.

(Fig. 5) and optimal growth on sucrose (Fig. 4A). It also restored wild-type citrate synthase activity (Fig. 4B). It seems that the *pcsA* gene confers on the bacteria a superior nodulation ability. This is supported by the fact that *R. leguminosarum* bv. *phaseoli* harbouring the *R. tropici pcsA* gene (Fig. 5, strain CFNE138) nodulates about 40% more and grows faster than the wild-type strain CFN42.

Gene expression regulated by iron was measured in CFN299 by the β-glucuronidase activity of a pBJ101.3 plasmid (Jefferson *et al.*, 1987) construct (which is stable in *Rhizobium*) carrying the *Pst*I–*Bam*HI fragment of *pcsA* (Fig. 1A, see the *Experimental procedures*). Beta-

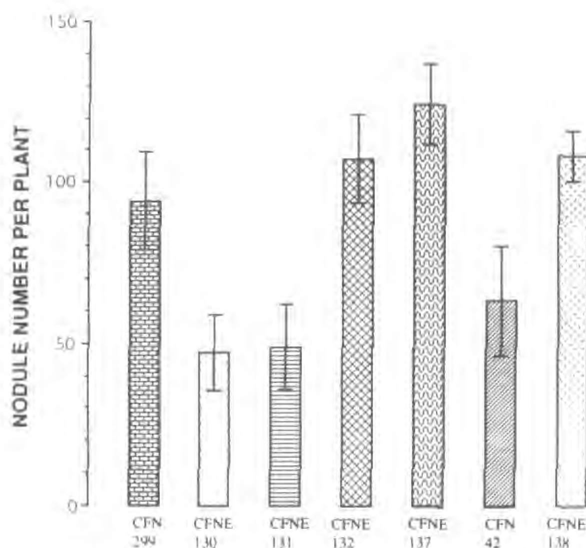

Fig. 5. *Nodulation abilities of: CFN299, R. tropici wild-type strain;* CFNE130, *pcsA* mutant; CFNE131, a CFNE130 rifampicin-resistant derivative, CFNE132, a CFNE131 with a wild-type pSym; CFNE137, a CFNE130 that contains a cloned wild-type *pcsA* gene; CFN42, *R. leguminosarum* bv. *phaseoli* wild-type strain; CFNE138, a CFN42 that harbours the cloned *pcsA* gene.

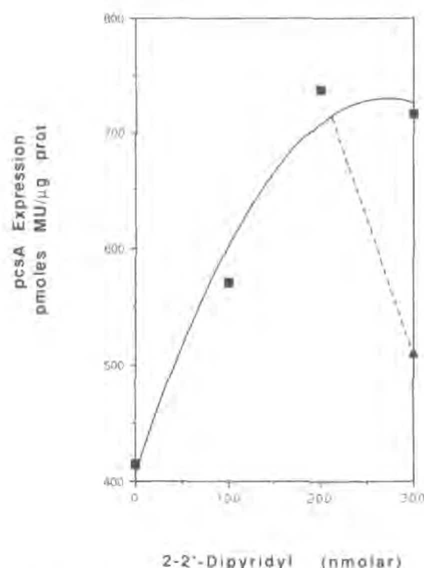

Fig. 6. *pcsA gene expression determined as β-glucuronidase* activity from CFN299 grown in decreasing iron concentrations. Plasmid pMP6 was transferred to wild-type strain CFN299. The transconjugant was grown in MM with increasing amounts of the iron chelator 2-2' dipyridyl. The dotted line and (▲) represent the condition where both 300 nM of dipyridyl and iron (1 μM) were added to the medium.

glucuronidase activity increased in proportion to the amount of the iron chelator 2-2' dipyridyl present in the medium (Fig. 6). In the wild-type strain, citrate synthase activity increased concomitantly with gene expression but not in the mutant strain CFNE130 (Fig. 7). Iron availability is a limiting factor for the growth of many microorganisms (Neilands, 1987) and in *Rhizobium*, iron acquisition is essential for nitrogen fixation. In iron-limited environments, bacteria produce siderophores to acquire iron. Citrate functions as an iron chelator in some *Bradyrhizobium japonicum* strains (Guerinot *et al.*, 1990), in *E. coli* (Hussein *et al.*, 1981), and in other microorganisms (Cox, 1980; Messenger and Ratledge, 1982;

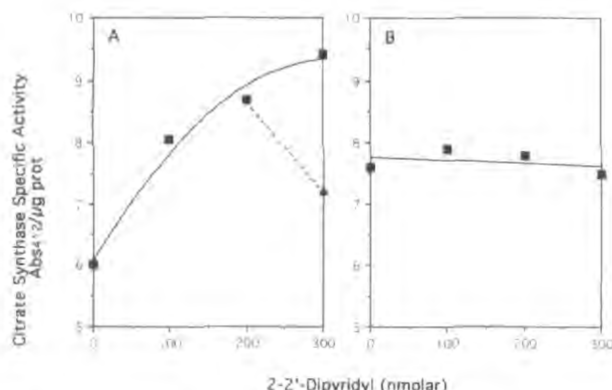

Fig. 7. Citrate synthase activity of (A) wild-type strain CFN299, and (B) the *pcsA* mutant CFNE130, with increasing amounts of dipyridyl in the medium. As in Fig. 6, the dotted line and (▲) represent a medium condition where both 300 nM of chelator and 1 μM of iron were added.

Ecker and Emery, 1983). It may also have the same role in *R. tropici*, as this bacteria is naturally found in acidic environments, where iron is not found in the hydroxylated forms. Citrate, which is considered to be a low iron-affinity siderophore may well serve as a chelator under these conditions. Complementation of the nodulation deficiency of CFNE130 was not restored by adding either ferric citrate or sodium citrate to the plant nodulation assays (data not shown): perhaps citrate is not reaching the bacteria in these assays during the nodulation process.

DNA duplication has long been recognized as an important factor in the evolution of new genes and genome size. Additional copies of chromosomal genes have been located in plasmids of *Rhizobium*. For example, in *Rhizobium meliloti*, *nodP* and *nodQ* are homologous to the *E. coli cysD* and *cysN* genes, whose gene products have ATP sulphurylase activity. *nodP* and *nodQ* are involved in the sulphation of the *R. meliloti* nodulation factor (Schwedock and Long, 1990). In *R. leguminosarum* bv. *viciae*, *nodM* is homologous to the *E. coli* housekeeping gene *glmS*, which codes for glucosamine synthase; both genes, *nodM* and *glmS*, have interchangeable functions (Marie *et al.*, 1992).

The biochemical functions of many plasmid-borne genes are largely unknown and this is also true for *Rhizobium* symbiotic plasmids. It is striking that a key metabolic enzyme such as citrate synthase is encoded on a symbiotic plasmid. A co-ordinated expression of the plasmid and chromosomal citrate synthase genes would be expected in order to avoid a bacterial metabolism collapse owing to an incapacity of the cell to control its carbon and energy flux. Our results show that the *Rhizobium* symbiosis should not be considered as an isolated bacterial process but rather as a whole bacterial metabolic adjustment to the host.

Notes added in proof

Interestingly, calcium limitation also stimulates *pcsA* gene expression as well as citrate synthase activity. This was not the case for other divalent cations. These data suggest that *R. tropici* may use citrate as a chelator to obtain iron and calcium, which are two important elements for the symbiotic process.

The American *Rhizobium leguminosarum* biovar *phaseoli* type 1 strains have recently been reclassified as *Rhizobium etli* (Segovia *et al.*, 1993).

Experimental procedures

Media and growth conditions

Minimal media (MM) was as described by Zaat *et al.* (1987), with 0.2% (w/v) of sucrose used as the carbon source. PY medium (complete medium) contained 0.5% peptone, 0.3% yeast extract and 7 mM of $CaCl_2$. Rhizobia were grown at 30°C.

Sequencing strategy

The Tn*5–mob* single insertion of strain CFNE130 was cloned in the *Eco*RI site of plasmid pUC18 and subclones were constructed in M13mp18 and M13mp19 (Yanisch-Perron *et al.*, 1985). The sequence of both strands was determined using the chain termination method (Sanger *et al.*, 1977) from overlapping clones shown in Fig. 1.

Plasmid profiles

Plasmids were visualized by the Eckhardt procedure (Eckhardt, 1978).

Enzyme assays

Beta-glucuronidase assay. Cells were sonicated in GUS extraction buffer (Jefferson, 1987) containing 1 mM 4-methyl umbelliferyl β-D-glucuronide (MUG). 4-methyl umbelliferone (MU), the product of β-glucuronidase activity, was measured spectrofluorometrically with excitation at 365 nm, and emission at 455 nm.

Citrate synthase assay. Cells were disrupted by sonication in Tris-HCl, 50 mM pH 8. Activity was measured spectrophotometrically at 412 nm by DTNB (5-5'-dithiobis(2-nitrobenzoate)) reduction according to the method of Halper and Srere (1977).

Hybridization conditions

Probes were labelled with ^{32}P by nick-translation (Rigby *et al.*, 1977). DNA was transferred from agarose gels to nitrocellulose filters as described by Southern (1975).

Assay for nodulation and nitrogen fixation

Nodulation assays were performed in agar flasks and in vermiculite jars using *P. vulgaris* Negro Jamapa as described

by Martínez *et al.* (1985), and Martínez-Romero and Rosenblueth (1990). Nitrogenase activity was measured by acetylene reduction.

Plasmid constructions

pMP6 was constructed by ligating the *Pst*I–*Bam*HI fragment (Fig. 1A) into the *Pst*I/*Bam*HI sites of the polylinker of plasmid pBJ101.3 (Jefferson *et al.*, 1987), which generates a translational fusion with GUS. Using the PCR procedure, the 2 kb *Pst*I–*Xba*I fragment containing the entire *pcsA* gene, 0.5 kb upstream and 0.2 kb downstream of *pcsA* (Fig. 1), was amplified and cloned into plasmid pRK7813 (Stanley *et al.*, 1987), generating plasmid pMP7. As pRK7813 can be maintained both in *E. coli* and *Rhizobium*, pMP7 was transferred by conjugation to the *pcsA*$^-$ *R. tropici* CFNE130 mutant (thereby generating strain CFNE137) and to *R. leguminosarum* bv. *phaseoli* CFN42 (thereby generating strain CFNE138).

Determination of genetic distances, sequence and phylogenetic analysis

Sequences were aligned using the program PILEUP from the Genetics Computer Group Sequence Analysis Package (Devereux, 1984). The Kimura-corrected distance was calculated for each pair of aligned sequences. Trees were obtained with the Fitch–Margoliash and Least Squares Methods, with an evolutionary clock using the program KITOCH87 from J. Felsentein's PHYLIP 3.4 package (Fitch and Margoliash, 1967).

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