Rhizobium tropici Chromosomal Citrate Synthase Gene

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Two genes encoding citrate synthase, a key enzyme in the Krebs cycle, have been found in *Rhizobium tropici*. One of them is in the bacterial chromosome, while the other is in the symbiotic plasmid. We sequenced the chromosomal gene and found that it is very similar to the previously reported plasmidic gene sequence in its structural region but not in its regulatory region. The chromosomal gene is able to complement an *Escherichia coli* citrate synthase mutant. In *R. tropici*, a mutant in the chromosomal citrate synthase gene has a diminished citrate synthase activity (in free-living bacteria), a diminished nodulation capacity, and forms nitrogen-fixing nodules. In contrast, the citrate synthase double mutant forms ineffective nodules devoid of bacteroids and forms less nodules than the single chromosomal mutant. It is inferred that both genes are functional and required during the nodulation process in *R. tropici*.

Citrate synthase catalyzes the condensation of acetyl coenzyme A and oxaloacetate to produce citrate and is considered the limiting step in the Krebs cycle (35). Its activity is important for carbon assimilation and for energy generation. In organisms such as *Bacillus subtilis* (10) and *Saccharomyces cerevisiae* (27), two citrate synthase genes are localized in the chromosome. In *Rhizobium tropici*, the nitrogen-fixing bacterium that nodulates *Phaseolus vulgaris* and other legumes (15), a citrate synthase gene is found in the bacterial chromosome (this work) and another is found in the symbiotic plasmid (19). The plasmid-borne gene (*pcsA*) has homology to the citrate synthase genes of protobacteria, and mutants of *pcsA* show a 30 to 50% decrease in nodule number compared with the wild-type strain (19).

In this study we addressed the question of the relative roles of the plasmid-borne gene and the chromosomal copy of the citrate synthase gene in *R. tropici* by analyzing the phenotypes of single or double mutants in free-living and symbiotically associated cells. In addition, a comparison of the nucleotide sequences of both genes showed that there is a large degree of conservation in the structural gene but not in the regulatory region. Our results support the hypothesis that these genes may be differentially expressed.

MATERIALS AND METHODS

Bacterial strains and media. The strains used in this study are listed in Table 1. Strains of *Escherichia coli* were grown in Luria broth, and *Rhizobium* strains were grown in PY medium (16) or in minimal medium (19) complemented with glutamate (200 mg/liter).

Cloning of the chromosomal citrate synthase gene of *R. tropici*. A DNA library was constructed from *R. tropici* CFNE 299-10, which has a deleted symbiotic plasmid and therefore lacks the *pcsA* gene. The genomic DNA was digested with *Sal*I, and fragments of between 1 and 2 kb were gel purified, ligated into the *Sal*I site of pBluescript SK⁺ (Stratagene), and transformed into *E. coli* DH5α (9). From the 1,250 transformants obtained, plasmids were isolated from 50 clones and analyzed for insert size after digestion with *Sal*I. All contained 1- to 2-kb fragments. This library was hybridized with *pcsA*. Four positive clones were obtained, three with a 1,628-bp insert and the other with a 1,208-bp insert.

Sequencing. The 1,628-bp (RT1) and 1,208-bp (RT2) clones and a PCR product (to fill the sequence gap between the two clones) were sequenced with synthetic primers by the method of Sanger et al. (29).

Citrate synthase mutants. Site-directed mutants were generated by double recombination of a 650-bp internal fragment of the *pcsA* gene interrupted with a *Bam*HI fragment from interposon pHP45 Ω carrying spectinomycin resistance (5). This construct was cloned in the vector pWS233 (31). Mutants were obtained after mating *E. coli* (*pcsA*::Ω) with strain CFNE 299-10 and selecting transconjugants in PY medium containing 7.5% sucrose, streptomycin (0.05 mg/liter), and spectinomycin (0.05 mg/liter), after which the mutants were tested for gentamicin sensitivity.

Hybridization conditions. DNA was purified as previously described (28) and transferred from agarose gels to nitrocellulose filters (32). Probes were labelled with ³²P by nick translation (25), and hybridization was carried out under high stringency conditions (28).

Assay for nodulation and nitrogen fixation. *Phaseolus vulgaris* cv. Negro Jamapa nodulation assays were performed in flasks with agar or vermiculite (13). Nitrogenase activity was measured by acetylene reduction 21 days after inoculation. Student's *t* test was performed to analyze the differences between nodule numbers in the different treatments.

Bacteroid isolation. Nodules from 6 to 10 plants harvested 23 and 28 days after inoculation were crushed in a mortar and filtered to eliminate debris as described previously (26). Bacteroids were isolated by centrifugation through Percoll gradients (23). The bacteroid fraction was collected, washed, and suspended in 1 ml of Tris-HCl (50 mM, pH 8) and sonicated three times for 1 min with 1-min rest periods. The homogenate was centrifuged, and the supernatant was used to measure citrate synthase activity.

Citrate synthase assays. Bacteroids or cells grown for 12 h in PY medium were harvested and disrupted by sonication as described above, and citrate synthase activity was measured spectrophotometrically at 412 nm by 5,5′-dithiobis-2-nitrobenzoic acid reduction (8). Citrate synthase activity measurements were performed at least three separate times for free-living bacteria. Determinations for bacteroids were performed twice in independent assays.

Sequence analysis and primers. Sequence alignments were carried out with the PC/Gene program (Intelligenetics, Inc., and Genofit, S.A.) and the gap program from the Genetics Computer Group program suite (3). We synthesized the citrate synthase chromosomal gene by PCR using total DNA from the CFNE 299-10 strain. The primers used were CTCGAATTCGTCGACGCCCGCCAA GTCTCG and CTCGAATTCAAACCGGCCATCTCTGACC. A 2.2-kb PCR product containing the whole chromosomal citrate synthase gene (*ccsA*) was cloned in vector pRK7813 (33) and used for the complementation assays.

Nucleotide sequence accession number. The sequence of the chromosomal citrate synthase gene is in the Genome Sequence Database under accession number L41815.

RESULTS

Chromosomal citrate synthase gene sequence. The sequencing strategy used to determine the complete nucleotide sequence of the chromosomal citrate synthase gene is presented

TABLE 1. Bacterial strains used in this study

Strain	Relevant characteristic(s)	Reference or source
R. tropici		
CFN 299	Wild type	15
CFNE 299-10	Sym plasmid deleted	14
CFNE 130	*pcsA1*::Tn5-*mob*	19
CFNE 140	*ccsA1*::Ω, sym plasmid deleted	This work
CFNE 150	*ccsA1 pcsA*$^+$	This work
CFNE 160	*ccsA1 pcsA1*	This work
CFNE 170	*ccsA1 pcsA1* (pRK7813 *ccsA*$^+$)	This work
E. coli		
DH5α		9
DH5α1	*pcsA2*::Ω	This work
MX1152	F$^-$ *gltA* mutant	17
MX1152(*ccsA*$^+$)	MX1152(pRK7813 *ccsA*$^+$)	This work

in Fig. 1. Two positive clones, RT1 and RT2, obtained from a genomic library, were partially sequenced in both strands. The internal citrate synthase gene fragment that lies between RT1 and RT2 was obtained by PCR synthesis with DNA from CFNE 299-10 and primers obtained from sequencing the RT1 and RT2 clones.

The coding region of *ccsA* is 90% identical to that of *pcsA* at the nucleotide level when the first ATG, 5 bp downstream from the chromosomal gene's Shine-Dalgarno sequence (Fig. 2A), is considered the protein initiation site. However, if the protein encoded by *pcsA* were smaller, starting at the ATG 114 bp downstream from the first ATG, then the identity between the two genes would be 98% at the nucleotide level. Most of the nucleotide differences between the two genes do not affect the primary structure of the protein, and there are no differences in the amino acids that are considered to be involved in the active site of the enzyme (Fig. 2B) (1, 36). In contrast, the regulatory regions of the two genes have only a 49.8% similarity (Fig. 2A).

Site-directed mutagenesis. The chromosomal citrate synthase gene of *R. tropici* was mutated in the CFNE 299-10 strain (lacking *pcsA*) by homogenotization with an interrupted *pcsA* fragment, as described in Materials and Methods. These mutants were hybridized with *pcsA*, and the hybridization pattern is shown in Fig. 3. The wild-type strain CFN 299 shows two hybridizing fragments, a 9-kb fragment corresponding to the chromosomal citrate synthase gene and a second band of 6 kb corresponding to the plasmidic gene. CFNE 299-10 has only the 9-kb band. The mutant strain CFNE 140 showed the expected 11-kb band because of the 2-kb interposon insertion in
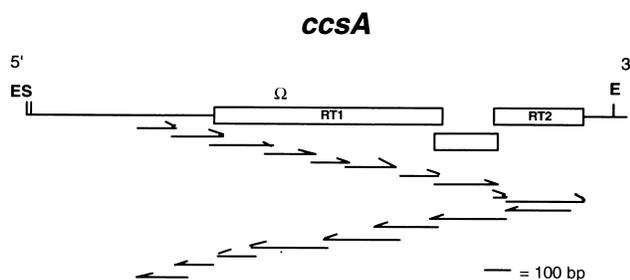
the 9-kb *ccsA*. A substantially lower citrate synthase activity was observed in this mutant (Table 2).

The symbiotic plasmid of the wild-type *R. tropici* strain CFN 299 was introduced by conjugation to the CFNE 140 strain to substitute a wild symbiotic plasmid for the deleted pSym, thus generating a CcsA$^-$ PcsA$^+$ mutant strain (CFNE 150). To obtain the citrate synthase double mutant (CFNE 160), the symbiotic plasmid carrying the mutated *pcsA1* gene from CFNE 130 was transferred to the CFNE 140 mutant strain. The double and the chromosomal mutants required glutamate for growth in minimal medium containing sucrose as the carbon source. In such conditions, mutants had growth rates identical to that of the wild type. Only a slight decrease in growth rate in minimal medium without glutamate was observed with the plasmid single mutant CFNE 130, as we have previously reported (19).

Genetic complementation analysis. A 2.2-kb PCR product containing the entire *ccsA* structural gene was introduced in an *E. coli gltA* mutant, MX1152 (17), and the transconjugant MX1152(*ccsA*$^+$) recovered the citrate synthase activity (Table 2). When the chromosomal citrate synthase gene was introduced in the *R. tropici* double mutant strain, CFNE 160, citrate synthase activity was recovered in the transconjugant strain, CFNE 170 (Table 2).

Symbiotic phenotypes of the citrate synthase mutants of *R. tropici*. Figure 4 shows the nodulation kinetics of the different citrate synthase mutants compared with the wild type. Both the chromosomal mutant (CFNE 150) and the double mutant (CFNE 160) have a large reduction in nodule number per plant; at 21 days these differences were highly significant ($P < 0.01$, by Student's *t* test), but no significant difference was detected between CFN 299 and CFNE 130, although CFNE 130 consistently showed a reduction in nodule number (Fig. 4). Nodules from the double mutant were white and did not fix nitrogen when measured by the acetylene reduction assay. From six plants analyzed, the average activity was almost undetectable, being 0.58% of the average activity obtained per plant nodulated by the wild-type CFN299. Furthermore, the microscopical analysis of nodules from the double mutant showed that they contained no bacteroids. The nodules obtained from both of the single mutants were Fix$^+$, with acetylene reduction values ranging from 30 to 40% of those obtained with the wild type. By microscopic analysis, they were seen to contain bacteroids (data not shown).

The transconjugant-complemented strain, CFNE 170, recovered its citrate synthase activity and formed the same number of nodules as the CFNE 130 strain.

Citrate synthase activity from bacteroids. Bacteroids from both of the single mutants CFNE 130 and CFNE 140 had reduced specific citrate synthase activities at two different times after inoculation (Table 2). Even though there was no visible bacteroid fraction in the double mutant, the corresponding sample was assayed and no citrate synthase activity was found. This also demonstrated that the plant citrate synthase activity was not contaminating the bacteroid fraction.

DISCUSSION

Citrate synthase genes from many organisms have been sequenced and reported. The chromosomal citrate synthase gene sequence of *R. tropici* is very similar to that of the *pcsA* gene that is located on the symbiotic plasmid. We suggest that the plasmid-borne gene resulted from a duplication of the chromosomal citrate synthase gene in *R. tropici*. In *Rhizobium* spp., there are other genes for which additional copies have been found in the genome; for example, *glmS* has a counterpart,

FIG. 1. Diagram of chromosomal citrate synthase gene (*ccsA*) showing Ω insertion to generate the *ccsA* mutant in *R. tropici*. The arrows delineate the extent and direction of the nucleotide sequencing procedure. Restriction sites: E, *Eco*RI; S, *Sal*I.

A

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       1                                                                                          100
ccsA   GACTCTCCACTCGGATCCGGTGCCTCTAAATTAGGCATACATCAAAGCGCGTACGCAAAACTACAGCTAAAGCGAGAGTTTTGCCGTCACTATTAGGTGA
pcsA   CCTG    GTT GCCGCTGTCAA A  GGCT  CTT  CC  CTC T  AGCGTATGTGCCGAG    C  TCTTG C C A  GATGA ACTGCC CCC

                                                                                                  200
       TTTCAACTGAAATCCGTCGGTTCTTCCATAAATGACGCCAATTCATCGTTGCGCAC......GGCAAGCCGGTCTACCGCATTGCCCAAAAGTTGATACT
       CCGG TGGATGC  G C TCA GG    A  CCATGCGG TGG TCG  CA  A  TTCAGGT A   TTT T GGT GT T GCT AT TC GCTCC

                                                                                                  300
       GCAATGCACAATTCAGCGTAAGGATAACTTGGCATTAGGCTCTGGATCATATAGCTAATCGTTAGTCGCTTAA.....CCCTTTGGCGCTTTTTAAGGCG
       TTCTCA GAGT GG    C CC CGA   CC    GCGGTT A CCCC  TGCGG AAG CAA     AACACAG TCAGTT A  C   AGC G   GTT C

                      ******                                                                       400
       CCAATCCCGCCAAATCTTGGAGGATCAGCATGACGGAACAAAGCGCGAAACTAACTTGGGGTGAAAAGACGGTGGATTTGCCGGTTAAGACCGAACCATC
        AGC AG  AGCCC GAG A AA  ATCATG  TAAC AC  TGCTTGTGTCTTAG C ACG CC TAGT CC A   GAAACT CG TCAA C  G

                                                                                                  500
       GGCCCAAGTGTCATTGATATTGGTGCCCTTTATAAGAACACCTCCACTTTTCACCTATGATCCTGGCTTCACTTCGACTGCGTCGTGCGAGTCCAGCATC
          T  G AC   C C GC    AT   C  CG C G  GAAG A.TG

                                                                                                  600
       ACCTTTATTGATGGCGACGAAGGCGTTCTGCTGCATCGCGGTTATCCGATCGAACAGCTTGCCGAGCACGGCGACTTCCTCGAAGTCTGTTACCTGCTGC

                                                                                                  700
       TCTACGGCGAATTGCCGACCGCAGCCCAGAAGAAGGACTTCGACTATCGGGTCGTGCACCACACCATGGTGCATGAACAAATGTCCCGCTTCTTCACCGG

                                                                                                  800
       TTTCCGCCGCGATGCGCATCCGATGGCCGTCATGTGCGGCTGCGTCGGCGCTCTGTCGGCCTTCTATCACGACTCGACCGACATCACCGATCCGCACCAG
                                                                       C

                                                                                                  900
       CGCATGGTCGCAAGCCTTCGTATGATCGCCAAGATGCCGACGCTTGCCGCCATGGCCTACAAGTACCATATCGGCCAGCCCTTCGTTTACCCGAAGAACG

                                                                                                  1000
       ATCTCGACTATGCATCGAACTTCCTGCGCATGTGCTTTGCCGTGCCCTGCGAGGAATATGTGGTCAATCCGGTGCTTGCCCGCGCCATGGACCGCATCTT
                G         T

                                                                                                  1100
       CATCCTGCACGCGGATCATGAGCAGAACGCATCGACCTCGACGGTTCGCCTTGCCGGCTCTTCCGGCGCCAATCCCTTTGCTTGCATCGCCGCCGGCATC
                    A                         C

                                                                                                  1200
       GCATGCCTCTGGGGGCCGGCCCATGGCGGCGCCAACGAAGCTGCGCTCAACATGCTAACGGAAATCGGCACGGTCGACCGCATTCCGGAATATATCGCCC
                    C                         CG              G

                                                                                                  1300
       GCGCCAAGGACAAGAACGATCCGTTCCGATTGATGGGCTTCGGTCATCGCGTTTACAAGAACTACGATCCGCGCGCCAAGATCATGCAGAAGACGGCGCA

                                                                                                  1400
       CGAGGTCCTCGGCGAACTCGGCATCAAGGACGATCCGCTGCTCGACATCGCGATCGAACTGGAGCGTATCGCCCTGACCGATGACTATTTCATCGAGAAG
                                                               G

                                                                                                  1500
       AAGCTTTACCCGAATGTCGACTTCTATTCCGGTATCACGCTGAAGGCGCTCGGCTTCCCCACGACCATGTTCACGGTACTGTTTGCGCTTGCCCGAACCG
                            C                                              A        C    C

                                                                                                  1600
       TCGGCTGGATTGCCCAGTGGAACGAGATGATCGAAGATCCGGATCAGCGTATCGGCCGCCCGCGCCACGTCTATACCGGCGCACCGCTGCGCGAATACGT
                                                                          GC

                                                                                                  1700
       TCCGGTTTCCAAGCGTTAAGC.CGAAAGGTCAGAGAGCAAAAACCCGGTCAGAGATGGCCGGGTTTTTTCCTGGATAAGACATCGAGAACGATCGCGCGC
                  C   A     C G  GAC TCC G TGA A G  T       CTGCTC GCTG  ATC CGCAGGGTTGA T CA  G C  C   A AGTG T

                 1771
       CTTTTGCCAGGCGCTCTTCGGTCTGCATTCGCTGCCAGACCAGATCGTAGCCTGAGCATGGCTGCAGCTCC
       GC  GA G AT TTG   CC TGCG AGGC  AG GCGAGCATG A  A GTT A A CGAC GTTCTG
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B

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        1                                                                                          100
ccsA    MTEQSAKLTWGEKTVDLPVKTEPSAQVSLILVPFIRTPPLFTYDPGFTSTASCESSITFIDGDEGVLLHRGYPIEQLAEHGDFLEVCYLLLYGELPTAAQ
pcsA    MDNNN CVLVDGHSAE KLRSSTIGPNV GIGSLYEQTKM

        101                                                        δ                               200
        KKDFDYRVVHHTMVHEQMSRFFTGFRRDAHPMAVMCGCVGALSAFYHDSTDITDPHQRMVASLRMIAKMPTLAAMAYKYHIGQPFVYPKNDLDYASNFLR

        201                                 δ                                     δ                 300
        MCFAVPCEEYVVNPVLARAMDRIFILHADEEQNASTSTVRLAGSSGANPFACIAAGIACLWGPAEGGANEAALNMLTEIGTVDRIPEYIARAKDKNDPFR
                                                                        R

        301    δ     δ      δ                                            δ     δ                 δ     400
        LMGFGHRVYKNYDPRAKIMQKTAHEVLGELGIKDDPLLDIAIELERIALTDDYFIEKKLYPNVDFYSGITLKALGFPTTMFTVLFALARTVGWIAQWNEM

        401      δ                      430
        IEDPDQRIGRPRHVYTGAPLREYVPVSKRF*
              QL          L        F
```
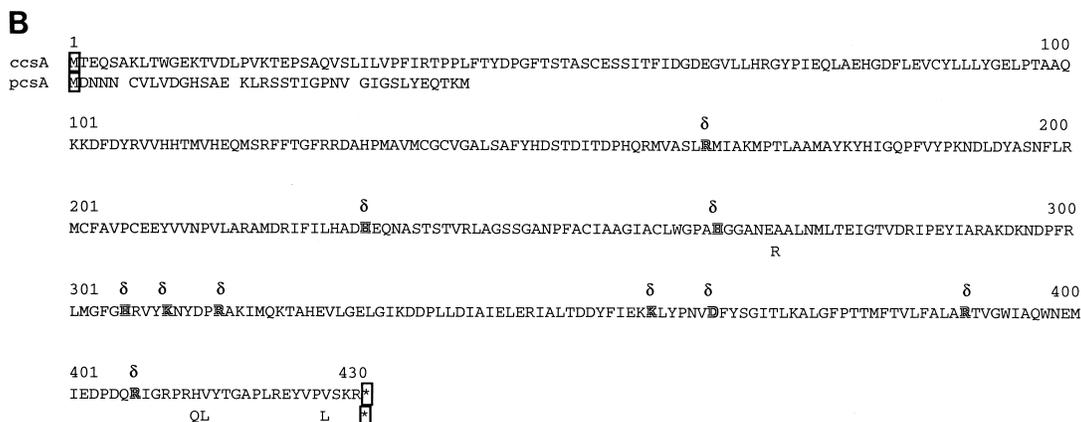
FIG. 2. (A) Chromosomal citrate synthase gene sequence and its comparison with the plasmid-borne gene. The nucleotides that are different are marked in the *pcsA* sequence. The putative Shine-Dalgarno sequence is marked with asterisks, the start and stop codons are underlined, and the putative terminal sequences are marked with dotted lines. The second ATG in the *pcsA* sequence is boxed. (B) Deduced amino acids of the chromosomal citrate synthase protein and comparison with the plasmid protein of *R. tropici*. The amino acids that are different are presented in the *pcsA* protein. Amino acids involved in substrate binding are outlined and marked with a δ. The starts and ends of the proteins are in boxes. Asterisks correspond to the stop codon of the gene.

nodM, that is specialized for symbiosis in *Rhizobium leguminosarum* (12); there are two copies of *nodPQ* (30) and two *fixN* regions in *Rhizobium meliloti* (24) and two *nifHDK* operons in *Rhizobium etli* (22). The additional copies of these genes may be the result of gene duplication. In the case of the *R. tropici* citrate synthase genes, the duplication seems to be ancient since the DNA sequence has clearly diverged outside the coding region, while there is a high degree of similarity in the coding regions of both genes. Besides duplication, there could be other explanations for the presence of *pcsA*; for example, lateral gene transfer between bacteria could have occurred and through recombination mechanisms with the chromosomal gene, the high degree of nucleotide similarity could have been obtained.

The promoter regions of the two genes are very different, and so the two genes could be regulated in different ways. In this respect, we could express the activity of only the chromosomal gene in bacteria grown in rich medium (PY) and in *E. coli*.

However, the contribution of each of these genes to the nodulation process is important. If either of the genes is present, the nodules formed are effective. This shows that both genes are involved in symbiosis and provide the required enzymatic products for nitrogen fixation. If the *ccsA* gene is mutated, a delay in nodulation is observed and only 30 to 40% as many nodules are produced relative to the wild-type strain (Fig. 4). In contrast, when *pcsA* is inactivated, 60 to 70% of nodules are produced without any delay in nodulation. It seems that the greatest activity, both in free life and in symbiosis, is provided by the chromosomal gene, while *pcsA* might constitute an acquired and specialized gene participating in the symbiotic process. This is supported by the fact that we detected citrate synthase activity in bacteroids in both of the single mutants (Table 2).

Delayed nodulation and ineffectiveness characterize the double mutant (CFNE 160). Similarly, in *R. meliloti* succinate dehydrogenase (7), α-ketoglutarate dehydrogenase (4) and isocitrate dehydrogenase (11) mutants also form ineffective
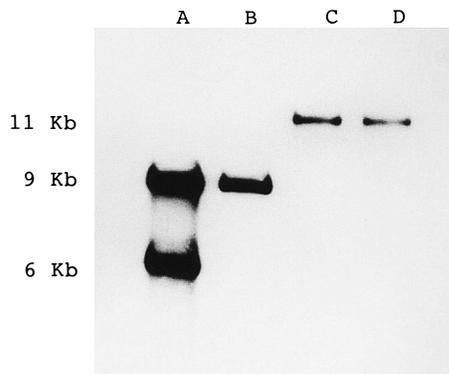
FIG. 3. Autoradiogram of *Eco*RI-digested genomic DNAs hybridized with the *pcsA* gene. The 6-kb hybridizing band corresponds to *pcsA*. The 9-kb hybridized band corresponds to *ccsA*. The 11-kb hybridized band corresponds to the *ccsA* pHP 45 Ω insertion mutant. Lanes: A, CFN 299; B, CFNE 299-10; and C and D, CFNE 140.

TABLE 2. Activities of citrate synthase genes from free-living bacteria and bacteroids

Strain	Activity (nmol/min/mg of protein)
E. coli	
MX1152	11[a]
MX1152(*ccsA*+)	178
DH5α	125
R. tropici	
CFN 299	210
CFNE 140	12[a]
CFNE 150	11[a]
CFNE 160	11[a]
CFNE 170	228
CFNE 299-10	206
CFNE130	207
Bacteroids	
CFN 299	128
CFNE 130	108
CFNE 150	75
CFNE 160	ND[b]

[a] Unspecific reaction.
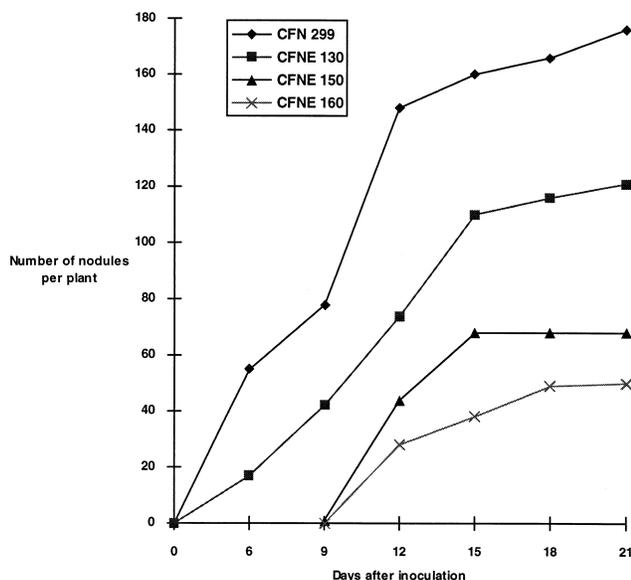[b] ND, not detected, since there is no bacteroid fraction with CFNE 160 (see the text).

FIG. 4. Kinetics of nodule formation in bean plants (*P. vulgaris*) elicited by *R. tropici* CFN 299 wild-type and citrate synthase mutants CFNE 130, CFNE 150, and CFNE 160. This experiment was repeated three times.

nodules on alfalfa. A complete Krebs cycle is needed for symbiosis, because the C4 dicarboxylic acids (succinate, malate, and fumarate), provided by the tricarboxylic acid cycle, constitute the best carbon source for bacteroids and therefore for an efficient symbiosis (2, 34).

The reduced nodulation capacity of the citrate synthase mutants may be a consequence of their auxotrophy, as they require glutamate for growth. It has been reported that citrate synthase mutants in other bacteria such as *E. coli* also require glutamate for growth (21).

Citrate synthase constitutes one of the limiting steps in the Krebs cycle and contributes to the formation of biosynthetic intermediates through pathways like the tricarboxylic acid and glyoxylate cycles (35). Citrate synthase has also been described as a regulator of *citB*, which codes for aconitase in *B. subtilis* (18). In *E. coli*, it seems to be repressed by *arcA* (20). It also seems to be involved in the acetic acid resistance in *Acetobacter aceti* (6). Previously we have shown that an efficient nodulation in *R. tropici* is conditioned by *pcsA*. All this supports the supposition that citrate synthase plays an important role as a housekeeping enzyme, but it may evolve and diverge to have different functions.

ADDENDUM

We found a second band of the citrate synthase gene in *R. tropici* type B CIAT 899[T]. In search of additional copies of citrate synthase in other *Rhizobium* species, we hybridized *R. etli* CFN42[T], *R. leguminosarum* biovar trifolii USDA 2046 and

USDA 2489, *R. meliloti* Rme2, *Rhizobium fredii* USDA 191, and *Agrobacterium* sp. strains K-Ag3 and Ch-Ag4, and only single bands were observed, indicating that most were probably unique citrate synthase copy genes. Two hybridizing bands were obtained with *Rhizobium galegae* 625 and *Rhizobium* sp. strain NGR234; it remains to be established if these bands correspond to additional citrate synthase genes and where they are located.

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