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# Identification of a system that allows a *Rhizobium tropici dctA* mutant to grow on succinate, but not on other $C_4$-dicarboxylates

**Silvia Batista, Ana I. Catalán, Ismael Hernández-Lucas, Esperanza Martínez-Romero, O. Mario Aguilar, and Gloria Martínez-Drets**

**Abstract**: A defined insertion mutant of a gene encoding a homolog of the rhizobial $C_4$-dicarboxylate permease (*dctA*) was constructed in *Rhizobium tropici* strain CIAT899. This mutant (GA1) was unable to grow on fumarate or malate; however, in contrast with other rhizobial *dctA* mutants, it retained a limited ability to grow on succinate with ammonia as a nitrogen source. Our results suggest the presence of a novel succinate-specific transport system in *R. tropici*. Biochemical characterization indicated that this alternative transport system in GA1 is active and dependent on an energized membrane. It was also induced by succinate and aspartate, and was repressed by glucose and glycerol. Bean plants inoculated with GA1 showed a reduced nitrogen-fixing ability, achieving only 29% of the acetylene reduction activity determined in CIAT899 strain nodules, 33 days after inoculation. Also, bean plants inoculated with GA1 had reduced shoot dry weight compared with plants inoculated with the wild-type strain.

*Key words*: succinate transport, *Rhizobium tropici*, $C_4$-dicarboxylate uptake.

**Résumé** : Un mutant insertionnel défini pour un gène codant pour un homologue de la $C_4$-dicarboxylate perméase des rhizobia (*dctA*) a été construit dans la souche CIAT899 de *Rhizobium tropici*. Ce mutant (GA1) était incapable de croître sur du fumarate ou du malate; cependant, contrairement aux autres mutants rhizobiens *dctA*, il a conservé une faculté limitée à croître sur du succinate en présence d'ammoniac comme source d'azote. Nos résultats indiquent la présence d'un nouveau système de transport spécifique au succinate dans *R. tropici*. La caractérisation biochimique a indiqué que ce système de transport alternatif dans GA1 est actif et dépendant d'une membrane activée. Il était également induit par le succinate, l'aspartate et réprimé par le glucose et le glycérol. Les plants de haricots inoculés avec GA1 ont montré une habileté réduite à fixer l'azote, atteignant seulement 29 % de l'activité de réduction de l'acétylène telle que déterminée dans les nodules de la souche CIAT899, 33 jours après inoculation. De plus, les plants de haricots inoculés avec GA1 avaient un poids sec de pousse inférieur comparativement aux plants inoculés avec la souche de type sauvage.

*Mots clés* : transport du succinate, *Rhizobium tropici*, incorporation du $C_4$-dicarboxylate.

[Traduit par la Rédaction]

## Introduction

The symbiotic association between rhizobia and leguminous plants requires that the reducing power and ATP needed to form and maintain nitrogen-fixing bacteroids are provided from plant photosynthates. Previous studies have indicated that $C_4$-dicarboxylates (DCA) are transported and metabolized by bacteria in the nodule as a major source of carbon and energy (Ronson et al. 1981; Gardiol et al. 1982). Mutants of *Sinorhizobium meliloti, Rhizobium leguminosarum,* and *Bradyrhizobium japonicum* incapable of DCA transport (*dct*), induce nodules containing ineffective bacteroids (Finan et al. 1983; Bolton et al. 1986; Engelke et al. 1987; El-Din 1992).

Biochemical characterization of DCA uptake has shown that the transport system of succinate is carrier-mediated, active, and generally inducible (Finan et al. 1981; McAllister and Lepo 1983; Bolton et al. 1986; Engelke et al. 1987). Succinate, fumarate, malate, and aspartate are transported by this system in *S. meliloti* (Watson 1990). In rhizobia the organization of the *dct* locus is conserved. The *dct* locus consists of a three gene cluster, the *dctA* gene that encodes a permease, and two regulatory genes (*dctB* and *dctD*). The *dctB* and *dctD* genes are found upstream of *dctA,* and are di-

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vergently transcribed (Ronson et al. 1984; Ronson et al. 1987; Yarosh et al. 1989). DctB and DctD are members of a family of two-component His-Asp-His-Asp phospho-relay environmental sensor-regulator genes (Nixon et al. 1986). The *dctB* and *dctD* gene products, together with sigma factor $\sigma^{54}$, are necessary for the expression of *dctA* under free-living conditions in response to the presence of DCA. The *dctBD* genes, however, are not required for nitrogen fixation. Nodules formed by *S. meliloti dctBD* mutants in association with *Medicago sativa*, were able to fix nitrogen at rates similar to the wild type, indicating that the *dctA* gene may be regulated by a distinct system during symbiosis (Engelke et al. 1989; Wang et al. 1989; Watson 1990). Boesten et al. (1998) found that DctBD-independent expression of *dctA* in *S. meliloti* is confined to the fixation zone (zone III) of mature nodules.

Bacteroid carbon metabolism has been characterized primarily in the rhizobia: *S. meliloti*, *R. leguminosarum* bv. *viciae*, and *B. japonicum*. The selection of these species to use as models for studies on bacteroid carbon metabolism, however, was done when the classification of *Rhizobium* species was based on the host plant that each strain was able to nodulate. In recent years there has been significant progress in the development of *Rhizobium* taxonomy due to the use of various DNA analysis methodologies, involving for example DNA–DNA hybridization studies, as well as ribosomal gene sequences and molecular marker analyses. Native rhizobia isolated from *Phaseolus vulgaris* and *Leucaena leucocephala* analyzed using these new methods enabled the identification of two new species of *Rhizobium*: *Rhizobium tropici* and *Rhizobium etli*. These new species were thus distinguished from *R. leguminosarum* bv. *phaseoli* (Martínez-Romero et al. 1991; Segovia et al. 1993). The strains belonging to *R. tropici* are distinct from the classical model systems discussed above in that they possess a relatively broad host range. *Rhizobium tropici* strains were able to form effective nodules on *P. vulgaris*, *Leucaena esculenta*, and *L. leucocephala*. Furthermore, using these same new methodologies, *R. tropici* was itself further subdivided into subgroups A and B. *Rhizobium tropici* was also found to be tolerant of environmental stress conditions like high temperature, acidity, and heavy metals compared with other species of *Rhizobium*. Strains of *R. tropici* may constitute a good model for extending previous studies on rhizobial carbon metabolism, in different hosts or under conditions of environmental stress.

The metabolic pathways in *R. tropici* (A; CFN299) which are active in the free-living form and during symbiosis were investigated by determination of enzyme activities involved in carbon metabolism (Romanov et al. 1994). Pentose phosphate pathway and Entner–Doudoroff pathway enzymatic activities were found to be present in sucrose-grown cells. The enzymatic activities found in bacteroids were similar to those found in malate-grown cells. In this paper we describe results of the biochemical and genetic characterization of a *dctA* mutant strain derived from the wild-type strain *R. tropici* CIAT899, and propose that in contrast to DCA uptake systems found in other species of *Rhizobium*, *R. tropici* CIAT899 may harbor a second *dctA*-independent system that alternatively recognizes and transports succinate.

## Materials and methods

### Bacterial strains, growth, and maintaining conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *Rhizobium tropici* cells were grown on PY medium (Noel et al. 1984) or on a minimal medium (MM) supplemented with 3 mM $CaCl_2$, 0.8 µM biotin, and 20 mM PIPES at pH 6.7 (Encarnación et al. 1995). *Sinorhizobium meliloti* cells were grown on a rich medium (RM) or on a defined medium (Batista et al. 1992). Both *Rhizobium* species were grown on minimal medium supplemented with nitrogen ($NH_4Cl$) and carbon sources at final concentrations of 10 mM and 20 mM respectively, unless otherwise indicated. *Escherichia coli* cells were grown in LB medium (Miller 1972). Antibiotics were added at the following concentrations: nalidixic acid (Nal), 50 mg/L; streptomycin (Sm), 50 mg/L; tetracycline (Tc), 10 mg/L; ampicillin (Amp), 100 mg/L; kanamycin (Km), 200 mg/L; gentamicin (Gm), 50 mg/L for *R. tropici* and 10 mg/L for *E. coli*.

Plasmids were transferred from *E. coli* S17-1 into *S. meliloti* and *R. tropici* by conjugation employing the method described by Simon et al. (1983) with some modifications. The mating mixture was suspended in 30 µL PY medium, then admitted onto a PY plate as a droplet. After overnight incubation at 30°C, transconjugants were selected on PY or RM media supplemented with the appropriate antibiotics.

### DNA methods

Cloning techniques, transformation of *E. coli*, isolation of plasmid DNA, and gel electrophoresis were done according to Ausubel et al. (1992). Restriction endonucleases and T4 ligase enzymes were used according to manufacturer's instructions (Promega Corporation, Madison, Wis.). Genomic DNA purification and hybridization procedures were done as described by Maniatis et al. (1982). Hybridization probes were labeled using the random-primed system, NEBlot-Phototope kit (New England Biolabs, Beverly, Mass.). PCR amplification of DNA from pRmSC121H6 was done using the oligonucleotides 5′CCACCCTCGCGCGCTTGTCG and 5′GCCCTCCCACTTCGCCACC, which are homologous to conserved nucleotide sequences located within the coding sequence of *S. meliloti dctA* as described previously by Engelke et al. (1989). The locations of these oligonucleotide sequences within the *dctA* gene of *S. meliloti* are at nucleotides 282 and 1238, respectively, with respect to the A of the start codon of the ORF encoding the predicted 46.1 kDa protein. For amplification, an initial denaturation for 2 min at 93°C, was followed by 34 cycles of 1 min at 93°C, 3 min at 45°C, and then 5 min at 72°C. The 100 µL PCR reaction mixture contained: 1× Taq polymerase buffer, 2.5 mM magnesium chloride, 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 0.05% (v/v) of Tween ×20 and Nonident P-40, 10 µM of each primer, 5 ng of DNA template, and 1 unit of Taq DNA polymerase (Promega Corporation).

A genomic cosmid library of *R. tropici* CIAT899 was constructed in pSUP205 (Table 1), a narrow host-range cosmid. DNA was partially digested with *Eco*RI (fragments with 25–35 kb size range), and cloned in pSUP205 linearized with *Eco*RI. The ligation mix was packaged into bacteriophage lambda heads for transfection of *E. coli* HB101. Random colonies were analyzed and found to have inserts of various sizes with an average size of 30 kb. Megaplasmid profiles from *R. tropici* and *S. meliloti* strains were analyzed by the Eckhardt method (1978).

DNA sequence analysis was done with an Applied Biosystems model 373A automated DNA sequencer (Perkin Elmer, Norwalk, Conn.), at the DNA Sequencing Core Laboratory at the University of Florida, Gainsville, Fla., U.S.A. The sequences of the complementary chains were confirmed using the original cosmid clone

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**Table 1.** Bacterial strains and plasmids used in this study.

| Strains and plasmids | Relevant characteristics | Source of reference |
|---|---|---|
| **Strains** | | |
| *E. coli* | | |
| HB101 | *supE44 hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1* | Beringer et al. 1978 |
| S17–1 | $Sm^r$ $Sp^r$ *hsdR* (RP4–2 *kan*::Tn7 *tet*::Mu, integrated in the chromosome) | Simon et al. 1983 |
| *R. tropici* | | |
| CIAT899 | Wild type $Nal^r$ $Sm^r$ | Graham et al. 1982 |
| GA1 | CIAT899 *dctA*::$Gm^r$ cassette | This work |
| CFN299 | Wild type | Noel et al. 1984 |
| *R. etli* | | |
| CFN42 | $Sm^r$ derivative | Segovia et al. 1993 |
| *S. meliloti* | | |
| RmF642 | Rm1021, *dctA14*::Tn5 | Yarosh et al. 1989 |
| RmF643 | Rm1021, *dctB20*::Tn5 | Labes and Finan 1993 |
| RmF644 | Rm1021, *dctB22*::Tn5 | Labes and Finan 1993 |
| RmF121 | Rm1021, *dctD16*::Tn5 | Yarosh et al. 1989 |
| U101 | RmL5–30, *dctA*::mini Mu | Batista et al. 1992 |
| **Plasmids** | | |
| pSUP205 | pBR325 derivative with *cos* site and RP4 *mob* site, $Cm^r$ $Tc^r$ | Simon et al. 1983 |
| pRmSC121H6 | pWKR561 with 6-kb *Hin*dIII fragment carrying *S. meliloti dct* genes | Engelke et al. 1989 |
| pRTD1 | pSUP205 derivative with *R. tropici dct* genes | This work |
| pBluescript II SK | Phagemid, M13 derivative, ColE1 replicon, $Amp^r$ | Stratagene |
| pRK767 | pRK747 derivative with pUC18 polylinker, $Tc^r$ a broad host range vector | Gill and Warren 1988 |
| pRK767-2A | pRK767 derivative with 4.4-kb *Eco*RI fragment from pRTD1, containing coding region of *R. tropici dctA* gene | This work |
| pAB2001 | pUC6S derivative with $Gm^r$ *lacZ* promotor-probe cassette, $Amp^r$ | Becker et al. 1995 |
| pWS233 | pSUP102 derivative with *sacRB* genes and *mob* site, $Gm^r$ $Tc^r$. | Selbitschka et al. 1993 |
| pWS233-2A | pWS233 with approx. 2.8-kb *Bgl*II-*Bam*HI fragment carrying *R. tropici dctA* gene and adjacent region cloned into *Bgl*II site. | This work |
| pTH24 | 6-kb *Hin*dIII fragment in pRK7813 polylinker | Yarosh et al. 1989 |

(pRTD1). The primers used for cosmid sequencing 5′GGCAGC-GATGGGAGCAAT (oligo 1) and 5′GCATTTGGCACGGCC-ATTGC (oligo 2), were synthesized by Life Technologies (Gaithersburg, Md.). The nucleotide sequence was translated by Gene Runner (Hastings Software) and searched against the nr protein database using the FASTA program. The DNA sequence of the *dct* region was also aligned with the corresponding fragments from *S. meliloti* and *R. leguminosarum* with Gene Runner.

### Determination of succinate and malate uptake activity

[$^{14}$C]succinate transport activity was measured in cells grown in the indicated medium to mid-exponential phase, harvested, and suspended in MM without a carbon source at O.D. = 7.0 at 620 nm. The succinate transport assay mixture, containing [2,3-$^{14}$C]succinic acid (159.5 μM; 8 μCi/μmol), unlabelled succinate (3.22 mM) and MM in 0.37 mL, was equilibrated in a bath at 30°C. The reaction was initiated by adding 0.25 mL of cell suspension ($4 \times 10^9$ c.f.u.) and samples were taken at indicated times and analyzed according to Batista et al. (1994). [U-$^{14}$C]malate transport activity was measured in cells grown in MM supplemented with 10 mM ammonium, 10 mM mannitol, and 10 mM succinate to mid-exponential phase. Transport assays were performed as described by Poole et al. (1985) with 100 μM unlabelled malate and [U-$^{14}$C]L-malate (55 mCi/mmol; 9 μM).

### Plant assay

Surface-sterilized seeds of *P. vulgaris* were inoculated with *R. tropici* and its mutant derivates, and grown in sterilized plastic pouches each containing Jensen medium (Vincent 1970). *Rhizobium* strains used as inocula were grown for 24 h in PY medium with the corresponding antibiotic, washed with MM without a nitrogen source and diluted to an O.D. of 1.0 at 620 nm prior to inoculation. Germinated seedlings were soaked in the cell suspension and incubated at 30°C for 2 h. The inoculated plants were then grown for 33 days, nitrogenase activity by the acetylene reduction assay (ARA) and shoot dry weights were then determined as previously described (Arias et al. 1979).

## Results and discussion

### Isolation and mapping of a *dct* locus from *R. tropici* CIAT899

A biochemical characterization of succinate uptake in *R. tropici* CIAT899 was reported previously (Batista et al.

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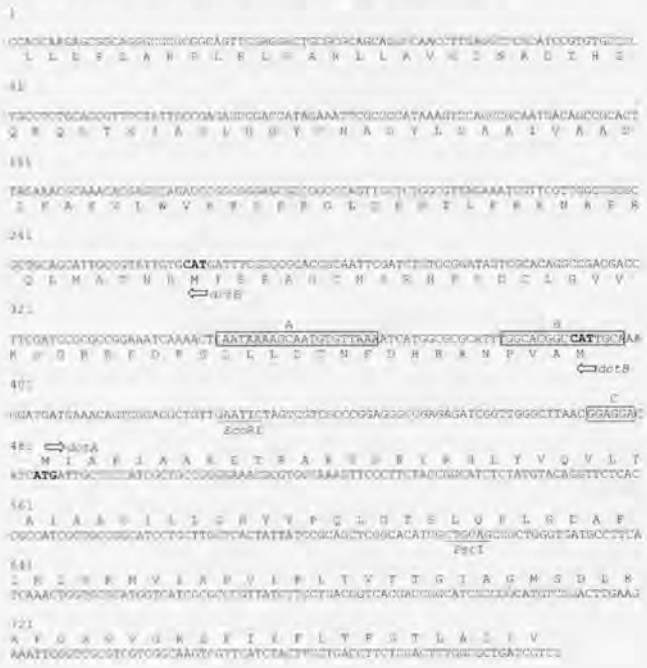
**Fig. 1.** Nucleotide sequence of the promotor regions of *R. tropici dct* genes. The ORF corresponding to the *dctA* gene is indicated by the deduced amino acid sequence written above the nucleotide sequence. The amino acids corresponding to the *dctB* gene, in the opposite direction, were written below the nucleotide sequence. The start codon indicated as *dctB′*, is located in a similar relative position of the *S. meliloti* and *R. leguminosarum dctB* gene start codons. Potential recognition sequences are indicated with boxes: A, DctB-binding site; B, RpoN-binding site; C, ribosomal binding site. *Pst*I and *Eco*RI restriction sites are underlined.



1994). The occurrence of an active system that depends upon an energized membrane was demonstrated. The uptake of succinate was induced in the presence of succinate, fumarate, and malate. In addition, fumarate or malate competitively inhibited succinate transport activity, suggesting the presence of a common-carrier system for these compounds. Overall, the succinate uptake system in *R. tropici* and other species of rhizobia appeared to be similar.

In order to identify a presumptive *R. tropici dct* locus, a $^{32}$P-labelled 6-kb *Hin*dIII DNA fragment from pRmSC121H6, which carries the *S. meliloti dctABD* genes, was used as a probe to screen a *R. tropici* genomic library (Table 1). Five cosmids containing *dctABD*-hybridizing sequences were obtained. Restriction mapping and hybridization analysis were used to identify two adjoining *Eco*RI fragments within these cosmids that encode a presumptive *dctABD*-like region. The *Eco*RI site between these fragments was later shown to be located in the intergenic region between genes that were found to be homologous of *dctA* and *dctB* (Fig. 1). The two *R. tropici Eco*RI DNA fragments (4.5 kb and 6.0 kb) from cosmid pRTD1 were subcloned into pBluescript II SK generating respectively subclones I and II. Subclone I, with the 4.5-kb *Eco*RI fragment, hybridized to a probe consisting of a 955 bp fragment obtained by PCR amplification of an internal fragment of the *S. meliloti dctA* gene (see Materials and methods). Subclone II, containing a 6.0-kb *Eco*RI fragment, hybridized to pRmSC121H6, but it did not hybridize to the 955 bp internal *dctA* fragment. Therefore, as it was found in other species of *Rhizobium*, the putative *dct* genes of *R. tropici* CIAT899 appear to be clustered.

Megaplasmid profiles from the bean-nodulating rhizobial species strains *R. tropici* CIAT899, CFN299, and *R. etli* CFN42 were hybridized to the *S. meliloti dct* locus, (probe from pRmSC121H6, see above), and found that in all cases the hybridization signal was associated only with the indigenous plasmids (unpublished results). In *R. tropici* CIAT899 the *dct* genes were located on a megaplasmid of around 1500 kb, using the 4.5-kb *Eco*RI fragment of Subclone I as hybridization probe. The *dct* locus of *S. meliloti* was also found on a plasmid as described previously (Watson 1990). Taken together, our results suggest that our putative *R. tropici* CIAT899 *dct* locus is organized in much the same way as the corresponding *dct* region of other rhizobia.

### Complementation of *S. meliloti dct* mutants

In order to assess the *dct* region for the ability to complement *S. meliloti dct* mutants, the 4.5-kb *Eco*RI fragment of *R. tropici* subclone I was recloned into plasmid vector pRK767 (Gill and Warren 1988) (Table 1). This subclone, designated pRK767-2A, was transferred by conjugation to *dct* mutant strains RmF642(*dctA*), RmF121(*dctD*), (Yarosh et al. 1989), RmF643(*dctB*), RmF644(*dctB*), (Labes and Finan 1993), and U101 (*dctA*), (Batista et al. 1992). It was found that this plasmid complemented not only the *dctA* mutant, but also other *dct* mutants affected in the regulatory genes *dctB* and *dctD*, respectively. This result was unexpected to us because only a small fragment from the 4.5-kb *Eco*RI fragment was found to have homology to the entire *dct* locus of *S. meliloti*. Further analysis of plasmid pRK767-2A revealed that cloning of the 4.5-kb *R. tropici* DNA into vector plasmid pRK767 had placed the *dctA* gene downstream of the p*lac* sequence. Therefore, expression of *dctA* was likely driven by the p*lac* and independently of the regulatory proteins DctB and DctD. Constitutive expression of *dctA* could explain the complementation of the *dctB* and *dctD* mutant strains.

### Sequence analysis of the *R. tropici dct*

Sequence analysis of the *R. tropici dct* region was done using the 4.5-kb and 6.0-kb *Eco*RI DNA fragments that were subcloned into pBluescript II SK (Table 1). Specifically, the sequence data was obtained from the region encompassing the junction of these two *Eco*RI fragments. Sequencing of both strands of pRTD1 was accomplished with appropriately designed primers. Analysis of the deduced amino acid sequence revealed two ORFs that are transcribed divergently. One of these ORFs, which starts at position nucleotide 484 in Fig. 1 had 75% identity with the DCA permease (*dctA* gene product) from *R. leguminosarum* (E3.1 e-32). Therefore, this ORF was assigned to be a potential *dctA* gene. A putative 16S ribosome-binding site and a potential recognition sequence for RpoN and DctD were identified upstream of the *dctA* coding region. The other ORF, which starts at position nucleotide 394, was found to have 48% identity with the DCA sensor protein (*dctB* gene product) from *S. meliloti* (E1.5 e-9). Therefore, this ORF was designated as

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**Fig. 2.** Construction of a pWS233 derivative plasmid containing partial sequences of the 2.8-kb *Bgl*II fragment isolated from pRK767-2A and a Gm$^r$-cassette derivated from pAB2001. This plasmid, named as pWS233-2A-Gm was employed to construct mutants by directed exchange mutagenesis. Abbreviations: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; P, *Pst*I.

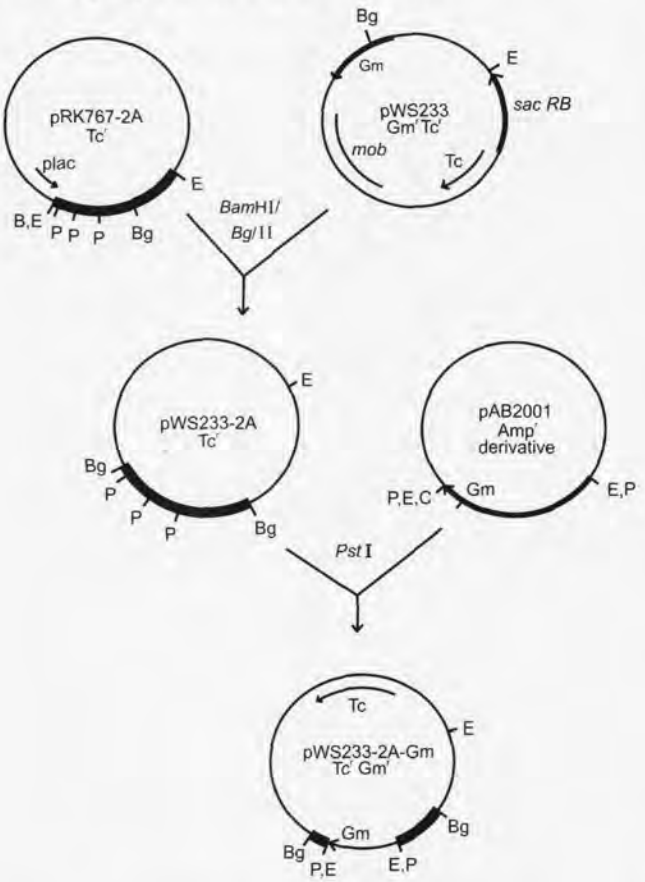
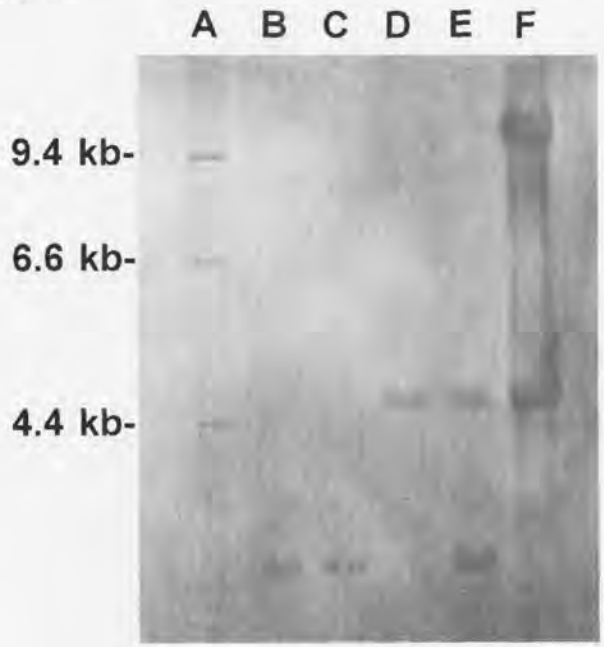



**Fig. 3.** Southern blot analysis of *R. tropici dctA* mutants. Total DNA from two double recombinant *dctA* mutants (Lanes B and C), wild-type strain (Lane D), wild-type strain with pWS233-2A-Gm cointegrated (Lane E) were digested with enzyme *Eco*RI and used to prepare Southern blots. pRK767-2A digested with *Eco*RI (Lane F) is also included. Hybridization was performed using 4.5-kb *Eco*RI *R.tropici* fragment isolated from pRK767-2A as probe, according to Materials and methods. Size markers from λ DNA digested with *Hin*dIII (Lane A) are indicated on the left of the figure.




a putative *dctB* gene. In this reading frame, however, another possible start codon was located 126 bp downstream of the ATG of *dctB*. The second ORF (*dctB'*) was found to have 48% identity with the DCA sensor protein from *S. meliloti* (E4.1 e-10). The second ATG, distal to the putative *dctA* gene, (at the beginning of ORF *dct* locus) is located in the same relative position of the *S. meliloti* and *S. leguminosarum dctB* start codons. The DNA sequence analysis of the 4.5-kb fragment confirmed that pRK767-2A encompasses the coding region of the presumptive *dctA* gene and a sequence of 57 bp upstream of the putative start codon. Restriction analysis confirmed that this gene was inserted in the same orientation of p*lac* promoter in the pRK767 vector (Fig. 1).

### Isolation of specific *R. tropici dctA* mutants

DNA sequence analysis of the *dctA*-region and restriction mapping revealed a *Pst*I site located 136 bp downstream from the initiation codon of the *dctA*–ORF. This site was then used to insert a part of the interposon (a 3.6-kb *Pst*I fragment) from plasmid pAB2001 (Gm$^r$, *lacZ*) (Becker et al. 1995). The 3.6-kb *Pst*I fragment is a modified version of this region of the interposon in that an internal *Cla*I fragment carrying a part of the *lacZ* reporter gene had been removed. The remaining portion of the interposon carries the gentamicin resistance gene.

The strategy that was used to construct the mutants is described below. The 4.5-kb *Eco*RI fragment (derived from Subclone I) containing the *dctA*-like coding region was subcloned in pRK767 (Table 1). Subsequently, a 2.8-kb *Bgl*II-*Bam*HI fragment (a *Bgl*II recognition site located within the 4.5-kb fragment and the *Bam*HI site located in the polylinker region of the vector plasmid) from this clone was inserted into the *Bgl*II site of pWS233 (Selbitschka et al. 1993) (Table 1) (pWS233-2A). This construction inactivates the gentamicin resistance gene of the plasmid, allowing the later use of gentamicin (present in the interposon) as selective marker for the isolation of a specific well-defined mutant. The resulting pWS233-2A was cleaved with *Pst*I and ligated to a 3.6-kb *Pst*I fragment containing part of the interposon (described above). There were two additional *Pst*I restriction sites found within the *Bam*HI/*Bgl*II-*Bgl*II *R. tropici* fragment of pWS233-2A. As such two internal *Pst*I fragments of about 500 and 800 bp were lost during the construction of pWS233-2A-Gm (see Fig. 2). The resulting construction contained 193 bp of the 5′ coding region of *dctA*, a deletion of 1.3 kb of the coding sequence (replaced with the interposon fragment), and 1.3 kb of the remaining *Bam*HI/*Bgl*II-*Bgl*II fragment. This plasmid, pWS233-2A, was then used to transform into *E. coli* S17.1 to construct a donor for subse-

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**Fig. 4.** Growth of *R. tropici* strains in different carbon sources. Cells were grown in PY for about 24 h, centrifuged, resuspended in MM supplemented with 10 mM $NH_4Cl$ and 20 mM of each of the substrates employed, except 1 mM Na glutamate. Cultures of CIAT899 (■), GA1 (○), GA1(pRTD1) (▲), and GA1(pTH24) (◇) were incubated in a rotatory shaker at 30°C.

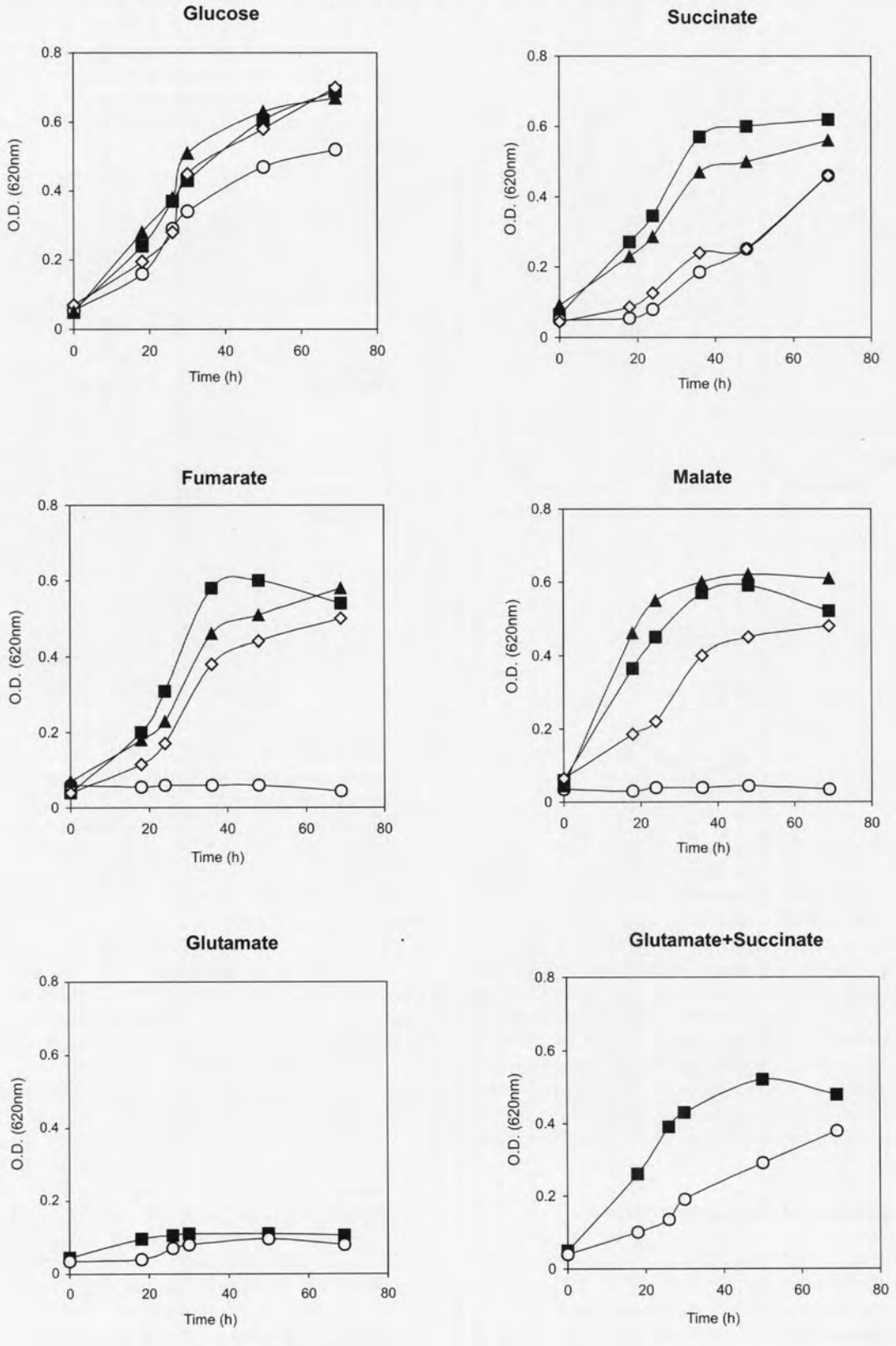


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**Table 2.** Effect of metabolic inhibitors on succinate uptake in GA1 strain.[a]

| Inhibitor[c] | Succinate uptake activity (%) |
|---|---|
| None[b] | 100 |
| NaCN (9.3 mM) | 14 |
| $NaH_2AsO_4$ (16 mM) | 110 |
| DNP (2 mM) | 18 |
| $Na_2EDTA$ (9 mM) | 115 |
| NaF (20 mM) | 63 |

[a]Cells were grown on MM with 20 mM succinate as carbon source. Cell suspension was preincubated in MM with the inhibitors for 4 min prior to the addition of a mixture of succinate and [$^{14}$C] succinate.
[b]Control succinate uptake rate was 31.4 nmoles·min$^{-1}$·mg protein$^{-1}$.
[c]Values in brackets are final concentration in succinate transport assay mixture.

**Table 3.** Induction of succinate transport in wild and mutant strain by different carbon source.

| Substrate in which cells were incubated | Succinate uptake rate (nmoles·min$^{-1}$·mg protein$^{-1}$) CIAT899 | GA1 |
|---|---|---|
| Glucose | — | — |
| Succinate | 17.0 | 56.0 |
| Fumarate | 5.2 | — |
| Malate | 14.8 | — |
| Aspartate | 5.4 | 7.4 |

**Note:** Cells were incubated in MM, with 10 mM ammonium with 10 mM of each substrate mentioned. —, undetectable levels.

quent conjugation and marker-exchange mutagenesis experiments. One *E. coli* S17.1 transformant was selected as a donor strain for conjugation with *R. tropici* CIAT899. Stable conjugal transfer of the plasmid to *R. tropici* CIAT899 requires integration into the chromosome since the plasmid is unable to replicate in rhizobia. Homologous recombination between the *dctA* regions of the remaining *Bam*HI/*Bgl*II-*Bgl*II fragment and the *R. tropici* CIAT899 chromosome should result from either cointegration of the entire plasmid or homogenotization of the marked deletion.

In order to isolate double recombinant *R. tropici dctA* mutants, an additional step of enrichment in MM–succinate supplemented with 1 mg/mL ampicillin was done. Fragment replacement in eight transconjugants that were found to be gentamicin resistant, tetracycline sensitive, and able to utilize succinate, was confirmed by Southern analysis (data not shown). The enrichment was done in MM–succinate with the presumption that *dctA* mutants should not be able to grow under these conditions. One of these mutants, designed strain GA1, was used for further analysis. Our subsequent finding that GA1 retained a limited ability to grow on succinate (see below) should be clarified, since one might expect GA1 to be counterselected during the enrichment. The enrichment step is of a relatively short duration (16 h), compared with the long lag phase which precedes growth of GA1 on succinate (see below). As such it is likely that the enrichment was of insufficient duration and thus preceded exponential growth of GA1. Hybridization analysis provided physical evidence that marker exchange had occurred in the *dctA* region of GA1. *Eco*RI digestion analysis of chromosomal DNA from the wild-type, GA1 and a predicted cointegrate yielded the expected results. As predicted, a 4.5-kb *Eco*RI fragment was evident in digests of the wild-type and a cointegrate, and a 3-kb fragment was present in the homogenote and cointegrate chromosomal DNA (Fig. 3).

### Growth properties of *dctA* mutant

The *dctA* mutant, designated as GA1, was found to be capable of growing on MM using ammonia or nitrate as nitrogen source and glucose or succinate as carbon source. GA1 could grow in MM–ammonia supplemented with succinate, but at a lower growth rate than the wild type strain and with a very long lag phase (Fig. 4). When the culture medium was supplemented, in addition to succinate, with 1 mM glutamate or 1 mM aspartate, the growth profile of strain GA1 was found to have a much shorter lag phase than without glutamate (Fig. 4) or aspartate (data not shown). Growth on DCA was restored when mutant GA1 was complemented by cosmid pRTD1, and by pTH24, respectively (Fig. 4). Moreover, GA1(pTH24) was able to grow on fumarate and malate as well as the wild-type. Growth of GA1(pTH24) on succinate as a carbon source, however, was only partially restored compared with that of the wild-type.

A similar result has been recently described by the isolation of *E. coli dctA* mutant (Davies et al. 1999). Under aerobic conditions, this mutant failed to grow on fumarate or malate, but retained a limited ability to grow on succinate. These authors suggested that *E. coli* could posses an alternative succinate transporter of relatively low activity that enabled *E. coli dctA* mutant to grow on succinate under aerobic conditions using SucT. Additional studies are necessary in order to fully understand the mechanisms underlying the alternative succinate transport system of *R. tropici* CIAT899, and its possible relationship to the SucT system.

*Rhizobium* sp. NGR234 harbors two systems for the transport of dicarboxylates: one located in the symbiotic plasmid (containing a homolog of the *dctA* gene) and another uncharacterized locus in the chromosome (Van Slooten et al. 1992). Although both systems were shown to be capable of DCA transport, only the one located in the symbiotic plasmid (pSym) was essential for nitrogen fixation in symbiosis.

Finally, Reid and Poole (1998) also showed that a *dctA* mutant of *R. leguminosarum* that expresses a low constitutive level of DctA was unable to grow on MM–succinate–ammonia unless the medium was supplemented with 10 mM glutamate or 10 mM aspartate. In that the growth properties of GA1 (a *dctA* null) are comparable, one might suggest that DctA may have additional functions besides that of a permease.

### Succinate and malate transport assay

[$^{14}$C]malate uptake activity in *R. tropici* CIAT899 cells grown in MM–ammonium–mannitol–succinate was 24.3 nmoles·min$^{-1}$·mg protein$^{-1}$. This activity was significantly higher than the one obtained with GA1 cells grown under the same conditions (0.95 nmol·min$^{-1}$·mg protein$^{-1}$). This dramatic difference illustrates that GA1 does not transport malate, and confirms that the *dct* system is responsible for the transport of malate in *R. tropici* CIAT899.

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**Table 4.** Symbiotic properties of *R. tropici* CIAT899 and *dct* mutant derivative.

| Strain or condition | Nodulation | ARA (μmoles $C^2$ $H^4 \cdot h^{-1} \cdot plant^{-1}$)[a] | Shoot dry weight ($g \cdot plant^{-1}$)[b] |
|---|---|---|---|
| CIAT899 | + | 14.0 | 0.21±0.06 |
| GA1 | + | 3.6 | 0.14±0.03 |
| with Nitrogen | — | 0.0 | 0.31±0.07 |
| without Nitrogen | — | 0.0 | 0.09±0.02 |

[a,b]Acetylene reduction activity (ARA) and shoot dry weight were performed on *P. vulgaris* plants 33 d after inoculation. Eight replicates were performed for each ARA determination. Eleven plants of each condition were employed for the shoot dry weight experiment.

GA1 cells grown on MM–succinate incorporated succinate linearly for at least 4 min, indicating the presence of a carrier-mediated system of transport. The concentration of succinate employed in the uptake assay was much higher than the usual 150 μM used and reported for other *dct* systems. The reason for this is because it was presumed that the alternative uptake system evident in GA1 is a low-affinity uptake system. The evidence for this is that in the presence of lower succinate concentrations (0.115 mM), the transport activity in the mutant decreased about 5 times (data not shown). This effect, however, was not observed in the wild-type strain. The succinate transport activity was maintained employing either 0.115 mM or 1.15 mM as the final concentration of succinate in the transport assay mixture.

In order to determine the presence of an active transport system, the effect of metabolic inhibitors was measured as described in Table 2. The activity of this transport system was strongly inhibited by metabolic uncouplers such as dinitrophenol (DNP) and cyanide, but not by arsenate and fluoride. Patterns of inhibition in *R. tropici* CIAT899 (Batista et al. 1994), and strain GA1 were found to be the same indicating that both the *dctA*–succinate uptake system and the alternative transport are active and dependent upon energized membranes. These results are consistent with those previously described for other *Rhizobium* species (McAllister and Lepo 1983; Hornez et al. 1989). Specificity of the succinate transport system in GA1 was examined in the presence of different substrates in the transport assay mixture. Malate and glutamate did not affect succinate transport activity, and fumarate showed a very small inhibitory effect, indicating a high level of specificity in this alternative system (data not shown).

Glucose or glycerol strongly repressed succinate uptake in GA1 (data not shown). As such, the regulation of the transport system that allows GA1 to grow on succinate was examined using cells previously grown in MM–ammonium–glucose. Bacteria grown in this medium were harvested and incubated in the presence of different substrates for about 24 h prior to analysis (Table 3). In agreement with other *dct* systems described previously, the succinate transport activity in *R. tropici* CIAT899 was induced by succinate, fumarate, aspartate, and malate. Glucose, fumarate and malate did not induce the activity of succinate transport in GA1. The transport activity in GA1 cells incubated in the presence of succinate was very high, compared with that of the wild-type strain. These results indicate a close interaction between the two transport systems, in that a regulatory network seems to not allow expression of the alternative transport activity in wild-type cells at the level observed in GA1.

Previous studies (Reid and Poole 1998) of R. leguminosarum have shown that in the absence of DctA, the DctBD two component system could "cross talk" with other unrelated systems and could also respond promiscuously to non-specific environmental cues. The absence of DctA in GA1 could be responsible for the expression of the alternative system in the dctA mutant. We suppose that in R. tropici CIAT899 the alternative system is not normally expressed, and may only be observed in dctA mutants. Further studies will be necessary to clarify this point.

The high level of succinate transport activity observed in GA1 should be correlated with a higher growth rate of GA1 in MM–ammonium–succinate, it should at least be similar to the growth rate of *R. tropici* CIAT899 under the same conditions. The absence of a correlation between the succinate transport activity and growth rate could be due to insufficient induction of Krebs cycle enzymes, or due to other elements that relate to the nature of this alternative transport system. A characterization of the carbon metabolism of *R. etli* indicated that a fermentative-like status can be achieved, and that the bacteria express specific transport systems required for the excretion of organic acids and amino acids into the medium (Encarnación et al. 1995). A transport system able to influx succinate and efflux organic acids could possibly explain the low growth rate observed in MM–ammonium–succinate for the strain GA1. Prior to doing the transport assays, bacteria are incubated under carbon and nitrogen deprivation, and then the succinate transport activity was measured. It should be acknowledged that the conditions of our transport assay differ from the conditions of bacterial growth in MM–ammonium–succinate, and that, for example, the amount of succinate incorporated during growth may differ from results obtained in our uptake assay. In this respect, it is important to note that the amount of succinate actually incorporated during growth may be conditioned by a potential efflux mechanism. More detailed experiments should be developed to characterize the alternative transport system and also to identify the factors involved in the expression of this alternative system.

### Symbiotic properties of *R. tropici dctA* mutant

The symbiotic properties of the *dctA* mutant strain GA1 were assessed in bean inoculation experiments. Thirty-three days after inoculation, nitrogenase activity of the mutant induced nodules was only 29% of that obtained by wild-type nodules (Table 4). Similarly, dry matter of the aerial parts of plants inoculated with mutant strain GA1 was lower than that of wild-type. The results obtained with the mutant GA1 differ from those found with other rhizobial *dctA* mutants (Engelke et al. 1989; El-Din 1992; Wang et al. 1989). We currently have no data that implicates the alternative system for a role in symbiosis. Measurement of dry matter of the aerial parts of plants inoculated with either *R. tropici*

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CIAT899 or strain GA1 after extended periods of time will be necessary to determine the possible role of the alternative system in the symbiosis.

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