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Two C_4-Dicarboxylate Transport Systems in *Rhizobium* sp. NGR234: Rhizobial Dicarboxylate Transport Is Essential for Nitrogen Fixation in Tropical Legume Symbioses

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To investigate the role of dicarboxylate transport in nitrogen-fixing symbioses between *Rhizobium* and tropical legumes, we made a molecular genetic analysis of the bacterial transport system in *Rhizobium* sp. NGR234. This broad host range strain fixes nitrogen in association with evolutionarily divergent legumes. Two dicarboxylate transport systems were cloned from *Rhizobium* NGR234. One locus was chromosomally located, whereas the other was carried on the symbiotic plasmid (pSym) and contained a *dctA* carrier protein gene, which was analyzed in detail. Although the DNA and derived amino acid sequences of the structural gene were substantially homologous to that of *R. meliloti*, its promoter sequence was quite distinct, and the upstream sequence also exhibited no homology to *dctB*, which is found at this position in *R. meliloti*. A site-directed internal deletion mutant in *dctA* of NGR234 exhibited a (unique) exclusively symbiotic phenotype that could grow on dicarboxylates *ex planta*, but could not fix nitrogen *in planta*. This phenotype was found for tested host plants of NGR234 with either determinate- or indeterminate-type nodules, confirming for the first time that symbiosis-specific uptake of dicarboxylates is a prerequisite for nitrogen fixation in tropical legume symbioses.

The *Rhizobium*-legume interaction results in an intracellular plant symbiosis in which the carbon-nitrogen metabolism of both partners becomes interdependent. Carbon and energy sources are supplied by plant photosynthate to the nitrogen-fixing *Rhizobium* bacteroids in the roots. The actual substrate has been postulated to be the C_4-dicarboxylic acids: succinate, malate, and fumarate (Bergersen and Turner 1967; Ronson *et al.* 1981). Mutants in C_4-dicarboxylate transport (*dct* mutants) of three temperate-zone rhizobia, *Rhizobium trifolii*, *R. leguminosarum* (Frank) Frank, and *R. meliloti* Dangeard, fail to fix nitrogen symbiotically in root nodules of their respective host plants (Ronson *et al.* 1984; Finan *et al.* 1981, 1983; Yarosh *et al.* 1989). These data also indicate that clover, pea, and alfalfa, temperate-zone legume host plants of these rhizobia, supply C_4-dicarboxylic acids to bacteroids to support effective nitrogen fixation. No such data are currently available for tropical legume symbioses, although they include agronomically important crop plants such as cowpea and soyabean. Because *Rhizobium* NGR234 fixes nitrogen with many evolutionarily divergent tropical legumes (Stanley and Cervantes 1991), the genetic analysis and mutation of its dicarboxylate transport system promised novel insights into the more general role of dicarboxylates in symbiotic nitrogen fixation.

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Nucleotide and/or amino acid sequence data are to be submitted to GenBank, EMBL, and DDBJ as accession number J0370B.

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In *R. leguminosarum* and *R. meliloti*, the *dct* regulon is contiguous and well-conserved between the species. Three *dct* genes are organized in two operons, *dctA* and *dctBD*, which are divergently transcribed (Ronson 1988; Yarosh *et al.* 1989). The regulatory genes *dctB* and *dctD* belong to a family of two-component regulatory genes: *dctB* codes for a periplasmic sensor of dicarboxylates, whereas the protein product of *dctD* is an activator of the transcription of the permease gene *dctA* (Ronson *et al.* 1987a,c). To clone Dct genes from *Rhizobium* NGR234, we used DNA hybridization with cloned *R. meliloti* and *R. leguminosarum dct* genes, and complementation of their *dct* mutants. We thereby cloned and characterized two quite distinct loci from *Rhizobium* NGR234. In one of these, we determined the nucleotide sequence of a *dctA* gene. The sequence of its promoter and upstream open reading frame (ORF) showed that the locus as a whole clearly differed from that of *R. meliloti*. A further novel feature of this NGR234 gene was its phenotype: the mutant was Dct^+ *ex planta*, but Fix^- *in planta*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Table 1. Complex (LB or TY) and defined (RM) media, and growth conditions were as described previously (Miller 1972; Beringer 1974; Stanley *et al.* 1989; van Slooten *et al.* 1990). RMS, RMF, and RMM were *Rhizobium* minimal media with 10 mM succinate, fumarate, or malate, respectively, as the sole carbon sources. Antibiotic concentrations used for *Escherichia coli* Migula (Castellani and Chalmers) and *Rhizobium* sp. NGR234 were as follows (μg/ml): Rif (50), Tet (10), Spc (50), and Gm (10).

Site-directed mutagenesis and general bacterial genetics. Allele replacement in NGR234 was performed as described (Stanley and Cervantes 1991) except that transconjugant NGR234 (pTVB60::Ω2) was subcultured six times before the incompatible "chaser" plasmid R751-pMG2 (Jacoby *et al.* 1976) was introduced. Ninety-four percent of tested transconjugants were Tet^s. Potential homogenotes were single-colony purified three times on minimal media before analysis by genomic Southern blots. General plasmid transfer and mobilizations for NGR234 were made as previously described (Stanley *et al.* 1989).

Table 1. Bacterial strains, plasmids, phage, and host plants used in this study

Strains, plasmids, phage, plants	Relevant characteristics	Origin or references
Bacterial strains		
Rhizobium sp.		
NGR234	Wild type	Trinick 1980
NGR234R	Rif^r, Nod$^+$, Fix$^+$	Stanley *et al.* 1987
NGR*dc*1	NGR234, *dctA*::ΔΩ1, Spc^r	This study
ANU265	NGR234, pSym-cured	Morrison *et al.* 1983
R. meliloti		
*Rm*1021	Su47, *str*21	Meade *et al.* 1982
*Rm*F642	*Rm*1021, *dctA*14::Tn*5*	Yarosh *et al.* 1989
*Rm*5421	*Rm*1021, *dctB*17::Tn*5*-233	Finan *et al.* 1988
*Rm*F121	*Rm*1021, *dctD*16::Tn*5*	Finan *et al.* 1988
*Rm*F726	*Rm*1021, Δ*dctABD*, *thi*::Tn*5*-233	T. M. Finan
*Rm*F728	*Rm*1021, Δ*dctABD*, *thi*::Tn*5*	T. M. Finan
R. leguminosarum		
*Rl*534	*dctA*::Tn*5*	C. W. Ronson
*Rl*535	*dctB*::Tn*5*	C. W. Ronson
*Rl*538	*dctD*::Tn*5*	C. W. Ronson
E. coli		
FM15R	F$^-$, Δ*lac-pro*, *thi*, *lacZ*, *recA*, Rif^r	Dowling *et al.* 1987
XL-1	*endA*, *hsdR*, *thi*, *recA*, Δ*lac*, (F′, *proAB*, *lacI*^{q}Z, ΔM15, Tn*10*)	Stratagene Inc., La Jolla, CA
Plasmids		
pRK7813	12 kpb, IncP1, Tc^r	Jones and Gutterson 1987
pRK7813-2	*Bam*-*Hin*dIII sites deleted from pRK7813	This study
pRK2013	*ori*V *col*E1, RK2-Tra$^+$, Km^r	Figurski and Helinsky 1979
pRK600	pRK2013 Km::Tn*9*, Cm^r	Finan *et al.* 1986
pBSM13$^{+/-}$	Phagemid, Amp^r	Stratagene Inc.
pMP220	IncP1, promoter probe	Spaink *et al.* 1987
pPN150	0.257 bp-f. *Rl dctA*	C. W. Ronson
pCR26	4.4 kb *Eco*RI-f., *Rl dctAB*	Ronson *et al.* 1984
pTH24	6 kb *Hin*dIII-f. *Rm dct*	Yarosh *et al.* 1989
pTVB60	NGR234 DctI locus, 5.8-kpb *Eco*RI-f.	This study
pHP45	Ω vector, Ap^{r}Sp^r	Frey and Krisch 1985
pTVB60::IΩ1	Ω inserted, *Bam*HI site	This study
pTVB60::ΔΩ1	Ω replaced *Cla*I-*Sst*I-f.	This study
pTVB60::ΔΩ2	Ω replaced *Sst*I-*Bam*HI-f.	This study
R751-pGM2	IncP1, Chaser, plasmid, Gm^r	Jacoby *et al.* 1976, Stanley and Cervantes 1991
pVSH1	pBSM13, DctA$^+$ *Hin*dIII-f.	This study
pMP220-S1	*Sma*I-f. of pVSH1, *dctA* promoter fusion	This study
pJS50	NGR234 DctII clone	This study
Phage		
R408	Helper/ssDNA rescue	Stratagene Inc.
Host plants		
Macroptilium atropurpureum	Family: Fabaceae, cv. Siratro	Duke 1981
Vigna unguiculata	ssp. unguiculata, Family: Facaceae, cv. Red Caloona	Duke 1981
Leucaena leucocephala	Family: Mimosaceae, cv. Cunningham	Duke 1981

Molecular cloning, DNA sequencing, and sequence analysis. The following were made by standard methods (Maniatis *et al.* 1982; Stanley *et al.* 1987): DNA preparation and digestion, agarose gel electrophoresis, Southern transfer, nick translation, hybridization of DNA digests, ligation, and bacterial transformation. Nucleotide sequences were determined by dideoxy chain termination (Sanger *et al.* 1977) after cloning DNA fragments into M13 mp18/mp19 (Yanisch-Perron *et al.* 1985) or the pBSM13+/- (Stratagene, La Jolla, CA) phagemid vector. DNA was sequenced as previously described (van Slooten *et al.* 1990) using Sequenase (Tabor and Richardson 1987) and ^{35}S-ATP (Amersham Corp., Arlingington Heights, IL). Overlapping contiguous clones were generated by specific cloning, and unidirectional deletions of double-stranded DNA were obtained by *Exo*III/Mung Bean nuclease (Stratagene) following the manufacturer's instructions.

Alignment and comparative analysis of nucleotide sequences were made with PC/Gene-NALIGN. Analysis of the sequence for ORF features was made with the programs PC/Gene-COD-FICK and TRANSL. Repeated nucleotide sequences in the promoter region were identified and analyzed with PC/Gene-REPEATS and hairpin loops with PC/Gene-HAIRPIN. Interspecific polypeptide homology comparisons of DCTA and its potential upstream ORF were made with PC/Gene-PALIGN, whose coordinates for similar amino acids were: Ser/Thr, Asp/Glu, Ile/Leu/Met/Val, Phe/Tyr, Arg/Lys/His, Gln/Asn, and Ala/Gly. Hydrophobicity analysis according to the method of Rao and Argos (1986) was made with PC/Gene RAOARGOS and according to the method of Klein *et al.* (1985) with PC/Gene-SOAP.

Plant tests. Nodulation tests and acetylene reduction assays were carried out 4 wk after inoculation of germinated seedlings. Plant growth conditions and assays were as previously described (Stanley *et al.* 1989; van Slooten *et al.* 1990).

RESULTS

Isolation and characterization of two Dct loci of NGR234. A partial *Eco*RI library of the NGR234 genome made in the vector pRK7813 was screened with the insert DNA of plasmid pCR26 (contains *dctA* and part of *dctB* cloned from *R. leguminosarum*). A homologous clone thereby isolated, termed pTVB60, contained one *Eco*RI fragment of 5.8 kb, corresponding to an *Eco*RI fragment of the same size that hybridized with pCR26 in the genomic DNA of NGR234R (data not shown).

pTVB60 was transferred to defined *dctA*::Tn*5* mutants of *R. leguminosarum* and *R. meliloti*. It complemented mutants *Rl*534 and *Rm*642 for growth on RMS, RMF, and RMM agars, and it similarly complemented *dctB* mutants (*Rl*535, *Rm*5421) and *dctD* mutants (*Rl*538, *Rm*F121) and was mapped with diverse restriction enzymes

(Fig. 1). By hybridization with pPN150 insert, a potential *dctA*-coding region was located on an internal *Sst*I-*Bam*HI fragment of pTVB60, and a corresponding fragment was located in genomic Southern blots. The interspecific complementation and hybridization data were taken to indicate that either three NGR234 alleles were present on the plasmid, or that the NGR234 *dctA* gene alone was present, but had a DCTB/D-independent mode of expression. This locus was termed DctI.

A second cloned NGR234 Dct locus was isolated via conjugation of the pRK7813 library of the NGR234 genome to *Rm*F121, selecting directly on RMS agar containing neomycin and streptomycin. These carried the unselected Tc^r marker of the clone vector and a recombinant plasmid termed pJS50. Plasmid pJS50 was retransferred to the mutants *Rm*5421, *Rm*642, *Rl*534, *Rl*535, and *Rl*538, and complemented all these for growth on RMS agar containing appropriate selective antibiotics. In both the original and the secondary complementation experiments, transconjugant Dct^+ colonies formed only after about 9 days of incubation, in contrast to Dct^+ complementation by pTVB60 in which the growth rate of transconjugants was the same as that of *Rm*1021 on RMS agar. Digests of pJS50 did not show interspecific homology with any *dct* probes, including pTVB60. We thus concluded that pJS50 encoded a functional Dct system of NGR234 that had no *dct* homology, and we termed it DctII. Partial *Eco*RI digestion products of pJS50 were subcloned, and its 7.1-kbp partial *Eco*RI subclone, termed pJS51, complemented all the previously tested individual *dct* allele mutants as well as *Rm*726, an *R. meliloti* mutant carrying a large deletion in the second *R. meliloti* megaplasmid that eliminates the entire *dct* operon and the linked *thi* gene. These pJS51 transconjugants also grew slowly initially (9 days), but upon subculture grew in 5 days on RMS agar. The restriction map of pJS51 is shown in Figure 1. Because the objective of the present study was to elucidate the contribution of rhizobial dicarboxylate transport to symbiotic nitrogen fixation by tropical legumes, the DctII locus was not further analyzed, other than its localization in the genome, due to our subsequent observation of the phenotype of a site-directed mutant in DctI.

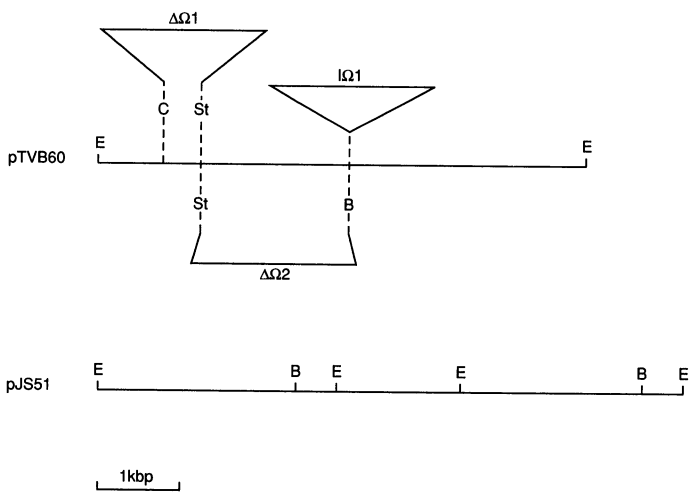

Fig. 1. Physical-genetic maps of the nonhomologous DctI and DctII loci of NGR234. Deletion (Δ) and insertion (I) mutants of pTVB60 (DctI clone) are shown. pJS51 (DctII clone) is also shown. See text for details of complementation analysis.

Nucleotide and deduced polypeptide sequences of *dctA* of NGR234. A physical map of the 5.8-kb *Eco*RI fragment carrying *dctA* of NGR234 is shown in Figure 2. The *Eco*RI-*Bam*HI fragment (leftward as drawn) that had complemented interspecific *dctA* mutants was sequenced first, using overlapping *Exo*III-Mung Bean nuclease deletions from the *Bam*HI site. Initial sequence data exhibited recognizable homology with the 3′ end of the *R. meliloti* gene, and the sequence was thus made inward from the NGR234 *Bam*HI site for both DNA strands (see Materials and Methods). All predicted ORFs were generated with the universal code, the initiation codons AUG/ATG or GUG/GTG and a minimum size of 10 amino acids. In the 1.7-kb *Sst*-*Bam*HI fragment, all phases of translation (confirmed start and termination codons) were consistent with only one complete ORF. The NGR234 *dctA* promoter was located on an *Hae*III-*Sph*I fragment, whereas the coding region of the gene was located on the contiguous *Sph*I-*Bam*HI fragment (Fig. 2). As shown in Figure 3, two translation-initiation codons were found (*dctA*1 and *dctA*2); and no strong ribosome-binding site was detected, although potential sites are indicated by asterisks. Alignment of the nucleotide sequence to the promoter region of *R. meliloti dctA* confirmed that in this area there was little identity (only 45% or 98 nucleotides). The 472-bp *Hae*III-*Sph*I (promoter) fragment contained a large number of repeated nucleotide sequences, some of which potentially encoded secondary structures such as hairpin loops. Eight direct repeats (between nucleotides 228 and 468) and 13 inverted repeats (between nucleotides 238 and 467) were detected. This should be noted in view of the unusual phenotype of the site-directed mutant (see below), which implied that the NGR234 *dctA* gene had an exclusively endosymbiotic phenotype.

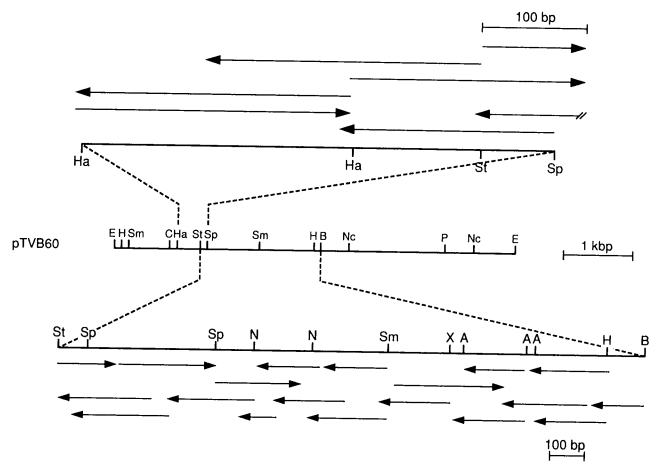

Fig. 2. Sequencing strategy for the NGR234 *dctA* gene and its promoter region. The *Eco*RI fragment of pTVB60 is shown at the center of the figure. The fragment containing the promoter is shown above this, and the *Sst*I-*Bam*HI fragment containing the structural gene is shown below this. The nucleotide sequence of the promoter was numbered from the first *Hae*III site to the *Sph*I site (nucleotide 474). Numbering of the *Sst*I-*Bam*HI fragment continues from the *Sst*I site to the *Bam*HI site at nucleotide 1,679. Arrows indicate strands sequenced after priming with universal or reverse primers. Restriction sites were as follows: *Alu*I (A), *Bam*HI (B), *Cla*I (C), *Eco*RI (E), *Hae*III (Ha), *Hin*dIII(H), *Nar*I (N), *Nco*I (Nc), *Pst*I (P), *Sma*I (Sm), *Sph*I (Sp), *Sst*I (St), and *Xho*I (X).

The region 5′ to the NGR234 *dctA* promoter (nucleotides 1–230) was found to be potentially transcribed divergently to *dctA*, with an initiation codon located at nucleotides 228–230, but lacked a detectable ribosome-binding site. This potential ORF would encode an N-terminal polypeptide of 76 amino acids with virtually no (8%) homology to DCTB, the product of the gene found in this area of the *R. meliloti* Dct regulon. The region downstream of the *dctA*-coding sequence (see below) contained no *rho*-independent terminator structures. The nucleotide sequence of the structural gene (with initiation codon *dctA*1, see below) was substantially (87%) homologous to the *R. meliloti dctA* gene sequence.

The nucleotide and the deduced polypeptide sequence of the gene are presented in Figure 3. The sequences from the initiation codons identified as *dctA*1 and *dctA*2 constituted 1,368 or 1,347 bp. They corresponded to polypeptides of 456 amino acids (NGRDCTA1; predicted molecular weight of 47.8 kDa) or 449 amino acids (NGRDCTA2; 47 kDa). On the basis of polypeptide homology with the *Rm*DCTA protein (see below), it was assumed that NGRDCTA1, which was strongly conserved with the *R. meliloti* protein (93% homologous, 88.7% identical, and 4.2% similar amino acids) was an NGR234 DCTA permease. Two regions of the polypeptide with least homology to *Rm*DCTA were N-terminal amino acids 2–21 and C-terminal amino acids 431–450. The deduced amino acid composition for NGRDCTA1 contained 71.9% hydrophobic residues and 28.1% polar residues, giving a probable isoelectric point (pI) of 8.72. Its domain hydrophobicity was analyzed by two computer programs (see Materials and Methods). The computer program of Rao and Argos (1986) detected six potential transmembrane domains capable of forming α-helices. They were amino acids 36–51, 65–90, 98–124, 175–193, 198–267, and 355–400. The computer program of Klein *et al.* (1985) detected the eight transmembrane domains that we show in Figure 3.

***dctA* gene expression analysis.** The genetic organization of the *dctA* locus, inferred from its DNA sequence, indicated that an active *dctA* promoter was located within the 1.9-kb *Sma*I fragment of pTVB60 (Fig. 2). This *Sma*I fragment contained the 5′ region of the upstream ORF, the promoter region of *dctA* and part of the *dctA* coding region. The *Sma*I fragment was converted into a *Bam*HI-*Eco*RI fragment by cloning it into and then excising it from the multiple cloning site of pUC19. The fragment was then cloned (5′→3′) between the *Bgl*II and *Eco*RI sites of the promoter probe vector pMP220. The recombinant plasmid, designated pMP220-S1, was transferred to NGR234 and to NGR*rn*3 (an *rpoN* mutant of NGR234).

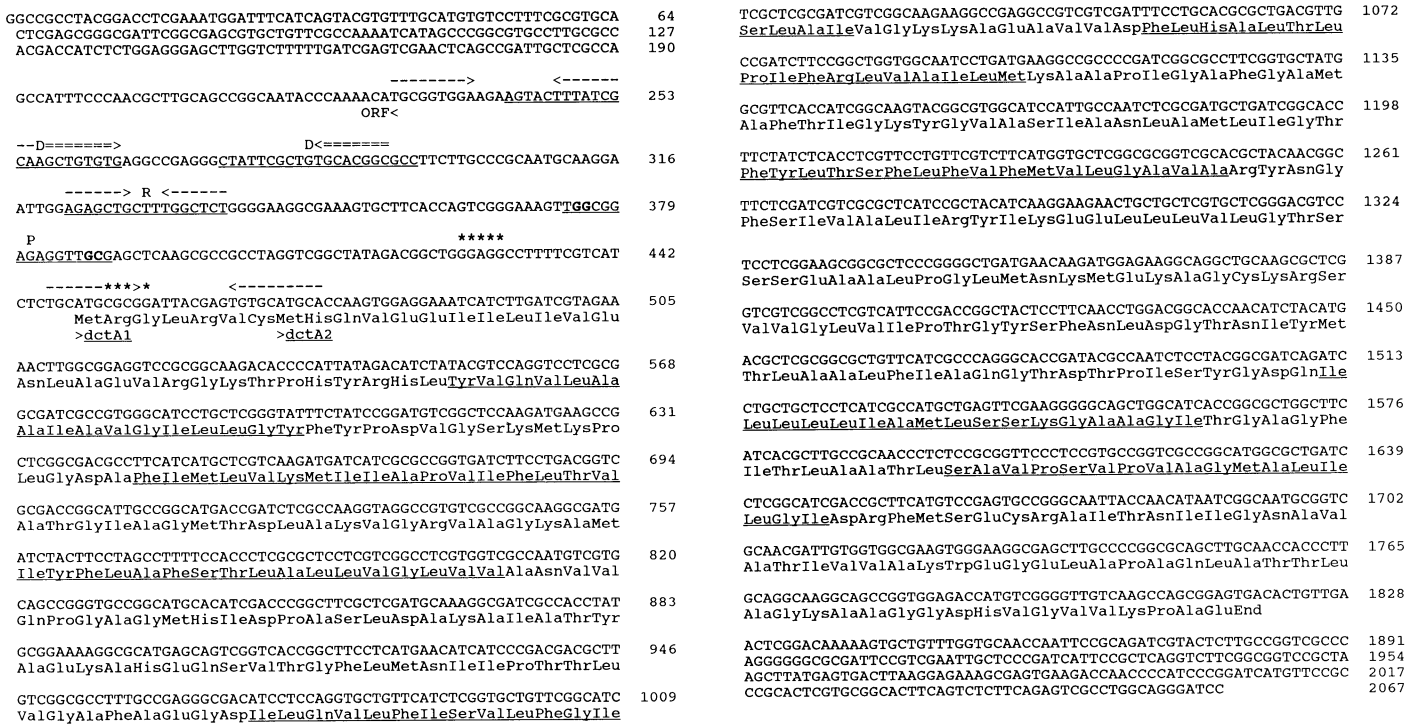

Fig. 3. Nucleotide and deduced amino acid sequences of the *Rhizobium* NGR234 *dctA* gene. Numbering of this sequence was made from the first (5′) *Hae*III site. In the promoter, three potential regulatory sequences (P, D, and R) are underlined. P is a probable RPON-consensus promoter; R is a potential regulatory sequence; D is a sequence homologous to the DCTD-binding domain of the *R. meliloti* locus (Jiang *et al.* 1990; Ledebur *et al.* 1990). The numbers at the right correspond to the last nucleotide of each line. The symbols <ORF, *dctA*1>, and *dctA*2> indicate the translation initiation sites of a divergently transcribed upstream open reading frame (ORF), and of the *dctA* gene. Asterisks indicate potential ribosome-binding sites. The arrows (→ or →→) indicate inverted repeats that are characteristic features of hairpin loops. Initiation codons *dctA*1 and *dctA*2 were found at nucleotides 449–451 and 1,817–1,819, whereas the termination codon was found at nucleotide 1,429. The deduced sequence of 456 amino acids was subjected to hydrophobicity analysis that identified eight transmembrane segments. As indicated by underlining between their first and last component amino acids these were: Tyr35-Tyr50; Phe66-Val82; Ile104-Val120; Ile175-Ile191; Phe202-Met218; Phe251-Ala267; Ile355-Ile371; and Ser384-Ile400.

pMP220-S1 produced high levels of β-galactosidase in NGR234, when expression was induced by growth on succinate. However, expression of β-galactosidase from the construct was noninducible in NGR*rn*3 (Table 2).

DctI (*dctA*) and DctII are located on pSym and the chromosome, respectively. Experiments were made to determine the location in the NGR234 genome of the loci cloned in pTVB60 (i.e., the *dctA* locus, termed DctI) and pJS50 (the second, functional locus, termed DctII). The *Bam*HI-*Sst*I fragment of pTVB60 (Fig. 2) that contained the *dctA* structural gene was employed as a probe against genomic DNA digests of NGR234 and its pSym-cured derivative ANU265. This (NGR234) probe identified a strongly and a weakly hybridizing fragment in the NGR234 genome under low stringency conditions of hybridization (6× SSC [1× = 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0], 10% dextran sulphate). In NGR234, an *Eco*RI fragment of 5.8 kbp hybridized strongly, whereas a 3.6-kbp fragment hybridized only weakly. The latter but not the former fragment was present in ANU265 (Fig. 4, A vs D). A *Hin*dIII fragment of 2.9 kbp hybridized strongly in NGR234, but was missing from ANU265. A *Hin*dIII fragment of about 8 kbp hybridized weakly and was present in both strains. Similarly, the 1.8-kbp *Bam*HI-*Sst*I fragment, self-hybridizing in the NGR234 genome, was absent from ANU265 (Fig. 2, C vs F).

These results were consistent with the location of the NGR234 *dctA* gene on an element specifically absent from the ANU265 genome (i.e., the Sym plasmid). The genomic fragment(s) of weaker homology observed with this (NGR234) probe in both the NGR234 and ANU265 genomes are therefore of chromosomal origin. With respect to size, those fragments did not correspond to *Eco*RI subfragments of pJS50 (which contains no *Hin*dIII sites). Conversely, when the DctII clone pJS51 was used to probe genomic DNA of NGR234, neither *dctA*-hybridizing fragment (e.g., *Eco*RI 5.8 and 3.6 kbp) showed homology. In both NGR234 and ANU265, pJS51 hybridized uniquely (data not shown) with genomic *Eco*RI fragments previously seen in its restriction map (see Fig. 1). Because it did not cross-hybridize with the 5.8-kbp *Eco*RI or 2.9-kbp *Hin*dIII *dctA* fragments identified in both genomes, it was deduced that the DctI and DctII loci of NGR234 were not significantly homologous at the nucleotide sequence level and that another locus with *dctA* homology existed in the NGR234 genome. The NGR234 *dctA* probe was next used to probe a canonical cosmid library of pSym NGR234 (Perret *et al.* 1991), and this library contained the requisite *Eco*RI, *Hin*dIII, and *Bam*HI-*Sst*I fragments previously established to hybridize strongly in total DNA of NGR234 (data not shown).

***dctA* of NGR234 is required for symbiotic nitrogen fixation in tropical legumes but has a cryptic phenotype *ex planta*.** The interposon Ω (Frey and Krisch 1985; Fellay *et al.* 1987) was cloned into the *Bam*HI site of pTVB60 (Fig. 1). The resulting recombinant plasmid, pTVB60::IΩ1, like pTVB60 itself, complemented *Rm*642 to a Dct$^+$ phenotype. On the other hand, when Ω replaced either the *Cla*I-*Sst*I fragment in the *dctA* promoter region or the *Sst*I-*Bam*HI fragment of the structural gene (Fig. 1), the resulting recombinant plasmids (pTVB60::ΔΩ1 and pTVB60::ΔΩ2) no longer complemented *Rm*642. pTVB60::ΔΩ2 was employed to generate a site-directed interposon mutant at the NGR234 *dctA* locus, as described in Materials and Methods. The allele replacement mutants were confirmed by genomic Southern blot analysis (data not shown), and one such site-directed mutant, deleted for the *Sst*I-*Bam*HI structural gene fragment, was termed NGR*dc*1. Surprisingly, and unlike reported *dctA* mutants of *R. meliloti* or *R. leguminosarum*, NGR*dc*1 was able to grow on RMS, RMM, and RMF minimal media containing dicarboxylates (succinate, malate, or fumarate) as sole carbon sources.

Table 2. β-Galactosidase activity from *dctA*::*lacZ* fusion pMP220-S1 in *Rhizobium*[a]

Strains	β-gal activity[b] on culture media (RM)	
	Succinate/glucose	Glucose
NGR234	$4^+/-2$	$5^+/-2$
NGR234 (pMP220)	$45^+/-5$	$45^+/-5$
NGR234 (pMP220-S1)	$110^+/-5$	$40^+/-5$
NGR*rn3*	$3^+/-2$	$3^+/-2$
NGR*rn3* (pMP220)	$40^+/-0$	$40^+/-0$
NGR*rn3* (pMP220-S1)	$30^+/-5$	$28^+/-5$

[a] *Rhizobium* strains were grown aerobically in RM and washed in carbon and nitrogen-free RM; 0.1% (v/v) was inoculated into RM containing 0.2% of the specific carbon sources. Five-milliliter cultures were shaken in 25-ml flasks at 30° C, and 150 rev/min. Growth was followed by measuring absorbance at 600 nm.

[b] β-Galactosidase was assayed as described by Miller (1972) after 48 hr for NGR234/NGR*rn3*. Each value is the average of three independent measurements, and standard errors are shown.

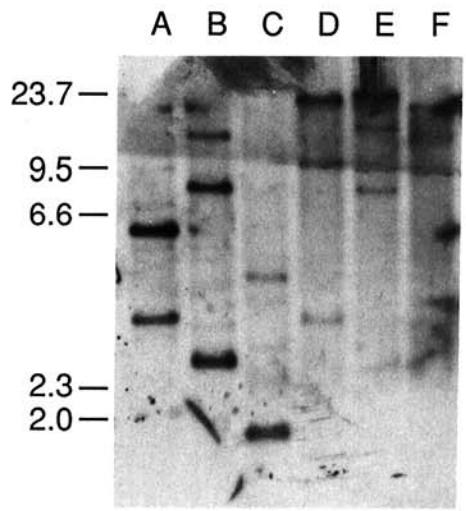

Fig. 4. Location of *dctA* on pSym of NGR234. Genomic DNAs of NGR234 and ANU265 were hybridized with the *Sst*I-*Bam*HI fragment internal to the *dctA* coding region of NGR234. Digestions shown from left to right are NGR234 **A,** with *Eco*RI; **B,** with *Hin*dIII; and **C,** with *Bam*HI-*Sst*I; followed by ANU265 **D,** with *Eco*RI; **E,** with *Hin*dIII; and **F,** with *Bam*HI-*Sst*I. *dctA*-specific hybridizing fragments in tracks A, B, and C (*Eco*RI, 5.8 kbp; *Hin*dIII, 2.9 kbp; *Bam*HI-*Sst*I, 1.8 kbp) are absent from tracks D, E, and F. The higher molecular weight fragments hybridizing in lanes B, C, and E, F and the fragment of approximately 3.5 kbp in lanes A, D do not represent the DctII locus (see text) but, rather, a third locus with homology to the intragenic *dctA* probe. Molecular size markers are shown at left.

This was also found for ANU265, the cured derivative that lacks pSym (and hence *dctA*). We considered that the observed phenotype was due to the presence, even in the NGR*dc*1 or ANU265 backgrounds, of the chromosomal dicarboxylate transport system, DctII, previously cloned in pJS51.

To evaluate the effects of the NGR*dc*1 (*dctA*) mutation on symbiotic nitrogen fixation, two determinate nodule (*Vigna unguiculata* (L.) Walp., *Macroptilium atropurpureum* (Moc. & Sessé ex DC.) Urb.) and one indeterminate nodule (*Leucaena leucocephala* (Lam.) de Wit) plants were compared in tests of symbiotic proficiency (see Materials and Methods). The results differed notably from those reported for this system in *R. meliloti*/ *R. leguminosarum*. Although NGR234*dc*1 was able to grow *ex planta* on RMS agar, it failed to fix any nitrogen in the three tested symbioses, as seen in Table 3. The data also confirmed that dicarboxylate transport by endosymbiotic rhizobia was indeed an essential feature of nitrogen fixation of these three tropical legume symbioses. We proceeded to make a comparative microscopic analysis of the Fix^- and Fix^+ nodules formed on *M. atropurpureum* by NGR*dc*1 and NGR234R. Cytological examination (Fig. 5) revealed that NGR234R formed nodules with a characteristic mosaic of infected and (few) uninfected cells. Uninfected cells contained prominent starch grains. On the other hand, NGR*dc*1 formed nodules which, although exhibiting characteristic determinate nodule morphology, had only few infected plant cells. The uninfected cells of these nodules contained no starch grains.

DISCUSSION

Genetic and molecular analysis of dicarboxylate transport by *Rhizobium* sp. NGR234 revealed two nonhomologous Dct loci. One was located on the symbiotic plasmid of NGR234 (DctI, containing *dctA*) and was required for symbiotic nitrogen fixation. The second was located on the chromosome (DctII) and could support growth on dicarboxylates, even of a site-directed *dctA* mutant, during the free-living state. The *dctA* gene of *R. meliloti* has been previously shown to be located on the pSym megaplasmid of that species (Watson *et al.* 1988), although its location was not specifically established in *R. leguminosarum*. The two rhizobial *dct* regulons that have previously been analyzed to the molecular level are those of *R. leguminosarum* (Ronson *et al.* 1987a) and *R. meliloti* (Wang *et al.* 1989; Jiang *et al.* 1989; Engelke *et al.* 1989; Watson 1990). By comparison, the DctI (*dctA*) locus of NGR234 exhibited several unique features that have not previously been reported for those regulons: it was not flanked by a *dctB* homologous gene; its promoter sequence (though not its coding sequence) was very divergent; and most importantly a site-directed mutant had an exclusively endosymbiotic phenotype.

Other than this, transcription of the NGR234 permease gene was observed to depend on the presence of a functional RPON sigma factor and was inducible by succinate, as is the case with *R. leguminosarum* (Ronson *et al.* 1987a) and *R. meliloti* (Ronson et al. 1987b; Yarosh *et al.* 1989).

Table 3. Symbiotic nitrogen fixation by *Rhizobium* sp. NGR234 and its *dctA* site-directed mutant[a]

Plants	NGR234R	NGR*dc*1
Macroptilium atropurpureum	6.3 (270)	<0.01 (20)
Vigna unguiculata	17.0 (1,760)	<0.01 (90)
Leucaena leucocephala	16.0 (350)	<0.01 (22)

[a] Acetylene-reduction assays were performed 35 and 45 days after inoculation for *Macroptilium* or *Vigna* and for *Leucaena*, respectively. Four plants were assayed per strain. Acetylene reduction data are given in micromoles of ethylene per hour per plant. The figure in parentheses indicates the average dry weight (mg) of plants at this time.

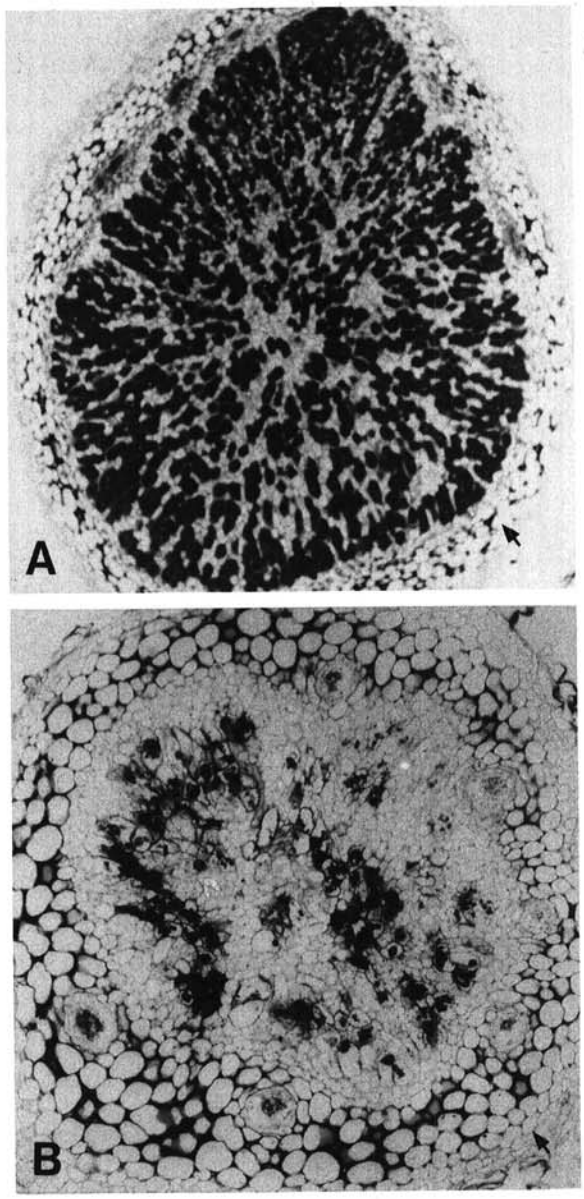

Fig. 5. Light microscopy of nodules formed on *Macroptilium atropurpureum*. An eight-micrometer section of nodules formed by **A,** NGR234 and **B,** its site-directed *dctA* mutant. **A,** Tissue map of wild type Fix^+ nodules showing nodule cortex (arrow) enclosing a mosaic of infected (dark areas) and uninfected cells (light areas). Magnification ×32. **B,** Tissue map of Fix^- nodules formed by mutant NGR234*dc*1. Nodules were smaller and contained a prominent cortex (↑), fewer infected cells (dark areas), and many uninfected cells. Magnification ×70.

The NGR234 promoter was a relatively weak one during *ex planta* growth. Although divergent in general nucleotide sequence (45% identity), the NGR234 *dctA* promoter shared several regions found in the *R. meliloti*/*R. leguminosarum* promoters: a potential site for a symbiotic activator (cf. Wang *et al.* 1989; Engelke *et al.* 1989; Ronson *et al.* 1987a), an Upstream Activator sequence (for DCTD in *R. meliloti*/*R. leguminosarum*; cf. Ledebur *et al.* 1990), and a promoter whose sequence conformed to that of the RPON consensus promoter (cf. Ronson *et al.* 1987a). This concurs with our previous demonstration that in NGR234, dicarboxylate transport is one of a number of symbiosis-related functions that are co-regulated by RPON (Stanley *et al.* 1989; van Slooten *et al.* 1990). DNA regions bound by regulators exhibit typical repeated regions capable of forming hairpin loops (Adhya 1989), and the hairpin loop structures found 5′ to the *dctA* gene of NGR234 are potential regulatory regions, currently of unknown function.

At 1,368 bp, the *dctA* gene of NGR234 was slightly larger than the corresponding ORF in *R. meliloti* (1,359 bp; Engelke *et al.* 1989) or *R. leguminosarum* (1,332 bp; Ronson *et al.* 1987a). NGR234 resembled *R. meliloti* in that a second initiation codon (*dctA*2) corresponded to the single one found in *R. leguminosarum*. In general, a substantial extent of structural gene homology was found for *dctA* at the nucleotide and amino acid sequence levels. The strong conservation of the gene implies that it encodes a product of functional importance for these rhizobia. Therefore, it is interesting that sequence conservation is also observed for essential symbiotic genes such as *rpoN*, *nodABC*, and *nifHDK*. The NGR234 DCTA permease was strongly hydrophobic throughout its whole deduced polypeptide sequence. Analysis of its hydrophobic domains (Kyte and Doolittle 1982) revealed six (method of Rao and Argos 1986) or eight transmembrane domains (method of Klein *et al.* 1985). Four of these were identical, and the last two domains deduced by the former method were split again by that of Klein *et al.* (1985). General analysis of DCTA can usefully be compared with that of *E. coli* lactose permease, which exhibits a similar transmembrane structure (cf. Foster *et al.* 1983). Our results concur with Tn*phoA*-mediated alkaline phosphatase fusion mutagenesis of *R. meliloti dct* genes which showed that *dctA* provided one of two secreted products for which transmembrane domains could be identified by *phoA* fusions (Long *et al* 1988; Yarosh *et al.* 1989).

The second DctII locus of NGR234 cloned was of chromosomal origin. The existence of such a second Dct system is consistent with two previous reports in the literature, which identified second Dct systems in rhizobia. First, two Dct systems were detected in *Bradyrhizobium japonicum* (Kirchner) Jordan by physiological criteria (Humbeck and Werner 1987). Second, an *R. meliloti* cosmid clone, nonhomologous to *dctABD* of that species, was isolated, which enhanced the rate of dicarboxylate transport and also of acetylene reduction by *B. japonicum*, a species capable of *ex planta* nitrogen fixation under microaerobic conditions (Birkenhead *et al.* 1988).

Our results show that a site-directed mutation deleting coding sequence from the *dctA* gene of *Rhizobium* NGR234 had no Dct$^-$ phenotype *ex planta*, but had a Fix$^-$ phenotype on all three host legumes tested, regardless of their nodule morphology type. Cytological examination of the determinate nodules formed on *Macroptilium* indicated that few plant cells were infected. By comparison, a *dctA* mutant of *R. meliloti* (Engelke *et al.* 1989) formed characteristic indeterminate alfalfa nodules containing bacteroids, but exhibited premature senescence. By light microscopic analysis of NGR*dc*1 nodules, we infer only that there was lower infectivity of nodule cells by the mutant. The low number of infected plant cells in *Macroptilium* nodules formed by NGR*dc*1 (Fig. 5) suggested that *dctA*-dependent use of host-supplied dicarboxylates may be obligate as early as the stage of intracellular release of rhizobia from the infection thread of these tropical legumes.

An important implication of our results with *dctA* of NGR234 is that the symbiosis-specific uptake of dicarboxylates is a prerequisite for nitrogen fixation in tropical legume symbioses. This was known to be the case for temperate-zone legume symbioses such as those of lucerne (cf. McRae *et al.* 1989), but bacterial genetic analysis has been lacking to date for dicarboxylate transport in rhizobia or bradyrhizobia, which are symbionts of tropical legumes. From the corresponding plant component of the symbiosis, a peribacteroid membrane-bound dicarboxylate transport protein has been characterized in nodules of soybean, a tropical legume (Udvardi *et al.* 1988).

In summary, we have described a *dctA* gene in *Rhizobium* NGR234, the mutation of which abolishes nitrogen fixation but is cryptic *ex planta*. A second Dct system exists in the same organism. The results extend to tropical legumes the general concept that dicarboxylic acids are the form of reduced carbon supplied by legumes to rhizobia to "drive" the symbiotic reduction of nitrogen.

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