Biochemical and Genetical Analysis of *Rhizobium meliloti* Mutants Defective in C₄-dicarboxylate Transport

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Inhibition studies with free-living cells of *Rhizobium meliloti* 2011 showed that succinate, fumarate and malate are transported via a common transport system. It is an active process that is inducible by succinate. The apparent K_m for succinate uptake in free-living cells is 5.3 μ M. Seven Tn5-induced mutants that did not grow on succinate, fumarate or malate lacked the C₄-dicarboxylate transport system in the free-living state (Dctfl⁻). Five of these mutants (RMS11, RMS16, RMS17, RMS24, RMS118) induced nodules on alfalfa with about half the nitrogen-fixation activity of the wild-type (Fix^{red}). The remaining two mutants (RMS420 and RMS938) gave rise to small, white and ineffective nodules on alfalfa (Fix⁻). These results correlated with the dicarboxylate transport rates of bacteroids isolated from appropriate nodules: bacteroids isolated from nodules induced by the Fix^{red} mutants transported succinate or malate at about 30–50% of the wild-type rate (Dcts^{red}), whereas bacteroids isolated from nodules induced by Fix⁻ mutants showed no uptake activity (Dcts⁻). From an *R. meliloti* 2011 bank three cosmids were identified which complemented different Dctfl⁻ mutants. Complemented Dctfl⁻ mutants could grow again on agar containing succinate or malate. In the case of Fix⁻ mutants complementation resulted in effective nodules. The cosmids pRmSC101 and pRmSC102 complemented the Fix⁻ mutant RMS420. A third cosmid, pRmSC121, complemented the second Fix⁻ mutant RMS938 and nearly all of the Fix^{red} mutants. For the Fix⁻ mutant RMS420 the Tn5 insertion site was found to be located on a 15.3 *Eco*RI fragment which is also part of the complementing cosmid pRmSC102.

INTRODUCTION

In the nitrogen-fixation process of the *Rhizobium*–legume symbiosis, a large amount of energy is required for the enzyme nitrogenase to reduce atmospheric nitrogen to ammonia. It is therefore of interest to identify the carbon compounds supplied by the plant and taken up by bacteroids. It is unlikely that sugars are used as energy source for nitrogen fixation because mutants of *R. leguminosarum* (Glenn *et al.*, 1984; McKay *et al.*, 1985), *R. meliloti* (Duncan, 1981) and '*R. trifolii*' (*R. leguminosarum* biovar *trifolii*) (Ronson & Primrose, 1979) defective in sugar metabolism are not affected in nitrogen fixation. Bacteroids of *R. leguminosarum* are also unable to transport glucose (Hudman & Glenn, 1980; De Vries *et al.*, 1982) or to oxidize it (Glenn & Dilworth, 1981). In addition, Saroso *et al.* (1986) reported the peribacteroid membrane of a cowpea *Rhizobium* strain to be relatively impermeable to sugars. On the other hand, a functional tricarboxylic acid cycle and an intact transport system for dicarboxylic acids seem to be important for effective nitrogen fixation. Mutants of *R. leguminosarum* (Finan *et al.*, 1981) and *R. meliloti* (Gardiol *et al.*, 1982) with a defective succinate dehydrogenase, and a further *R. meliloti* mutant with a defective 2-oxoglutarate dehydrogenase (Duncan & Fraenkel, 1979)

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Abbreviation: DNP, dinitrophenol.

induced ineffective nodules. Mutants of *R. leguminosarum* (Glenn *et al.*, 1980; Finan *et al.*, 1981, 1983; Arwas *et al.*, 1985) and two *Bradyrhizobium* species (San Francisco & Jacobson, 1985), unable to transport succinate, fumarate or malate in the bacteroid state, did not show nitrogen-fixation activity. In contrast, a few mutants were found which had no transport activity in the free-living condition but still fixed nitrogen in the symbiotic state (Finan *et al.*, 1983; Arwas *et al.*, 1985). *R. meliloti* mutants (Bolton *et al.*, 1986) and '*R. trifolii*' mutants (Ronson *et al.*, 1981) that were unable to transport succinate in the free-living state were also unable to induce an effective symbiosis with alfalfa.

In this paper we describe investigations of the dicarboxylate transport system of *R. meliloti* in both the free-living and the symbiotic state, and report the isolation of Tn5-induced mutants to find out how nitrogen fixation in *R. meliloti* is dependent upon a functional C₄-dicarboxylate transport system.

METHODS

Bacterial strains and plasmids. These are listed in Table 1.

Media and growth conditions. *Escherichia coli* cells were grown in LB medium (1% w/v, Bacto-tryptone, 0.5% yeast extract and 0.5% NaCl), in LBMM medium (LB plus 4 g MgCl₂ and 1 g maltose l⁻¹), in Penassay broth or in TY medium (0.5% Bacto-tryptone, 0.3% yeast extract and 0.1% CaCl₂), at 37 °C unless otherwise mentioned. *R. meliloti* cells were grown in TY medium or in minimal medium at 30 °C. *Rhizobium* minimal medium contained: 12 mM-K₂HPO₄, 11 mM-KH₂PO₄, 2.5 mM-NaCl, 6.3 mM-NH₄NO₃, 2.0 mM-MgSO₄, 70 µM-CaCl₂, plus 1 mg each of biotin, thiamin and calcium pantothenate l⁻¹ (pH, 6.5). Biotin, thiamin and calcium pantothenate were filter-sterilized separately. Carbon substrates, all filter-sterilized, were added at a final concentration of 10 mM. Growth rates were estimated by measuring OD₅₈₀ (Kontron Uvikon 820). For growth on solid medium 1.5% (w/v) agar was added. Antibiotics were used at the following concentrations: ampicillin (Ap) 50 µg ml⁻¹, tetracycline (Tc) 5 µg ml⁻¹, chloramphenicol (Cm) 50 µg ml⁻¹, kanamycin (Km) 25 µg ml⁻¹, neomycin (Nm) 100 µg ml⁻¹ and streptomycin (Sm) 400 µg ml⁻¹.

Plant assays. Alfalfa seeds were surface-sterilized with 32% (v/v) HCl for 15 min, then washed twice with 100 ml 70% (v/v) ethanol and three times with 100 ml sterile H₂O. The seeds were incubated in the dark for 36 h in Petri dishes on nitrogen-free medium which contained: 1.2 mM-K₂HPO₄, 0.4 mM-KH₂PO₄, 5 mM-CaCl₂, 3.35 mg ferric citrate l⁻¹, 2.5 mM-MgSO₄, 2.5 mM-K₂SO₄, 10 µM-MnSO₄, 20 µM-H₃BO₃, 5 µM-ZnSO₄, 0.2 µM-CuSO₄, 1.5 µM-CaSO₄, 1.0 µM-NaMoO₄ and 10 g agar l⁻¹ (pH, 6.8). Three seedlings were transferred to each fresh Petri dish containing alfalfa growth medium which had been preinoculated with about 10⁸ bacteria. After 24 d incubation at 20 °C and 70% humidity, plants were harvested and incubated with acetylene. After 1 h, acetylene reduction capacity was measured by gas chromatography (Hewlett Packard 5710A).

Preparation of bacteroids. The procedure used is a modification of that described by Finan *et al.* (1983). Nodules were harvested from 21-d-old plants. In the case of effective nodules 40 plants, and in the case of ineffective nodules 120 plants, were usually sufficient to obtain enough nodule material. The harvested nodules were placed in 10 ml cold MMS buffer (40 mM-MOPS, 20 mM-KOH, 2 mM-MgSO₄ and 300 mM-sucrose, pH 6.5, and homogenized in a mortar to release the bacteroids. The homogenate was filtered through eight layers of

Table 1. *Bacterial strains and plasmids*

Strain	Relevant characteristics	Reference
<i>E. coli</i> SM10	<i>thi thr leu suIII recA</i> Km ^r , containing RP4-2(Tc::Mu) integrated into the chromosome	Simon <i>et al.</i> (1983)
<i>E. coli</i> K802	<i>hsdR hsdM gal met supE</i>	Maniatis <i>et al.</i> (1982)
<i>E. coli</i> S605	<i>thi thr leu suIII lac met::Tn5</i> Km ^r	R. Simon, Bielefeld
<i>E. coli</i> S17.1	<i>pro Res⁻ Mod⁺ recA</i> , containing RP4 integrated into the chromosome	Simon <i>et al.</i> (1983)
<i>R. meliloti</i> 2011	Sm ^r	J. Denarie, France
pSUP205	Cm ^r Tc ^r , a pBR325 derivative containing the λ cos site and the RP4 Mob site	Simon <i>et al.</i> (1983)
pSUP2021	Ap ^r Cm ^r Nm ^r , a pBR325 derivative	Simon <i>et al.</i> (1983)
pSUP5011	A pBR325 derivative containing Tn5 and the RP4 Mob site	Simon <i>et al.</i> (1983)
pNC206	Ap ^r Km ^r , a derivative of RP4	D. Haas, ETH Zürich

cheesecloth and centrifuged at 400 g for 10 min to sediment plant cells and cell fragments. The supernatant was centrifuged at 2000 g for 10 min. The resulting pellet contained the bacteroid fraction as judged by microscopic examination. This fraction was washed three times with transport buffer (see below). The whole procedure was carried out at 4 °C.

Uptake experiments. The method used is a modification of that described by Finan *et al.* (1981). An exponential-phase culture of free-living *R. meliloti* was centrifuged at 4000 g. The cells were washed, like the bacteroids, three times with cold (4 °C) transport buffer (4 mM-MgSO₄, 20 mM-NH₄Cl, 0.1 mM-KH₂PO₄, 1.2 mM-K₂HPO₄, 20 mM-KOH, 0.2 mM-CaCO₃, 40 mM-MOPS, pH 6.5) and resuspended in fresh transport buffer (4 °C). The sample was incubated at 30 °C for 10 min before adding [2,3-¹⁴C]succinate (Amersham). In experiments in which 2,4-dinitrophenol (DNP) was present, cells were preincubated for 10 min at 30 °C with DNP before adding [¹⁴C]succinate. Usually, at time intervals of 20 s, 100 µl culture samples were withdrawn, vacuum-filtered using nitrocellulose filters with a pore size of 0.45 µm (Sartorius), and washed with 5 ml cold transport buffer. The filters containing cells were dried at 100 °C for 30 min and resuspended in 5 ml scintillation fluid containing 3.5 g PPO and 0.2 g POPOP per litre toluene. ¹⁴C radioactivity was measured in a scintillation counter (Hewlett Packard Tri-Carb 300). Non-specific binding of [¹⁴C]succinate to the outer cell wall was estimated by incubating the reaction mixture with 2% (v/v) toluene for 30 min at 30 °C prior to the addition of [¹⁴C]succinate. Protein concentration was measured by the Lowry method with lysozyme (Serva) as a standard.

Recombinant DNA methods. Cosmid DNA from *E. coli* was prepared on a large scale by a caesium chloride centrifugation essentially as described by Clewell & Helinski (1969) or on a small scale by the rapid isolation procedure described by Ish-Horowitz & Burke (1981) with the following modification. The supernatant, before ethanol precipitation, was treated with 0.44% diethyl pyrocarbonate (Sigma) for 15 min at 65 °C to inactivate the residual proteins. After incubation, the mixture was microfuged for 2 min and the supernatant was transferred to a fresh tube and ethanol precipitated. The *E. coli* transformation procedure, isolation of *Rhizobium* DNA, agarose gel electrophoresis and Southern hybridization have been described before (Jagadish & Szalay, 1984). Restriction enzymes (*Eco*RI, *Hind*III) were used according to the manufacturer's specifications.

A cosmid gene bank of *R. meliloti* 2011, constructed in *E. coli* K802 using the cosmid vector pSUP205, was available (Hynes *et al.*, 1986).

Bacterial matings for transfer of plasmid/cosmid DNA. *E. coli* and *Rhizobium* cells were grown in liquid medium to late-exponential phase, mixed in a 1:1 ratio in Eppendorf tubes, microfuged and resuspended in 25–50 µl TY medium. Portions (20–30 µl) of this suspension were spotted onto sterile filters (Sartorius) placed on TY agar. After incubation at 30 °C for 15–24 h, the filters were resuspended in 1 ml sterile water if the recipient was *R. meliloti*, or 1 ml fresh TY medium if the recipient was *E. coli*. Undiluted or serially diluted samples were plated onto appropriate selective plates (containing tetracycline and streptomycin) for the growth of transconjugants.

A cosmid gene bank stored as a λ lysate was transfected into *E. coli* S17.1 in the following way: 0.1 ml lysate was added to 5 ml of *E. coli* S17.1 strain grown in LBMM to exponential phase, mixed and incubated at 37 °C with gentle shaking for 30 min. *E. coli* S17.1 now containing the cosmid bank was mated *en masse* to the *R. meliloti* mutants as described above.

Isolation of *R. meliloti* dicarboxylate transport mutants by Tn5 mutagenesis. The Tn5 mutagenesis system developed by Simon (Simon *et al.*, 1983; Simon, 1984) was used. *E. coli* SM10, containing RP4-2(Tc::Mu) integrated into the chromosome (to provide Tra⁺ functions during mating) and a plasmid (pSUP2021) carrying Tn5 (Nm^rKm^r), was mated with *R. meliloti* 2011 (Sm^r). Transconjugants were selected on TY agar plates containing neomycin and streptomycin. The Sm^rNm^r transconjugants were subsequently screened in parallel on *Rhizobium* minimal medium containing glucose (10 mM) or succinate (10 mM) to select for mutants that grew on glucose but not on succinate. This procedure excluded mutants defective in amino acid or nucleotide biosynthetic pathways.

Isolation of cosmid clones that complement C₄-dicarboxylate transport mutants. A cosmid gene bank of *R. meliloti* 2011 in pSUP205 (stored in *E. coli* K802 or as a λ lysate) was used for the isolation of clones able to complement the *R. meliloti* dicarboxylate transport mutants. This was achieved by mobilizing the cosmids into *E. coli* using IncP-group plasmid RP4 and transferring them into *R. meliloti*. Several pooled batches of the bank containing RP4 (cells were Tc^r due to the cosmid vector pSUP205 and Ap^r due to RP4) were mated *en masse* with the *R. meliloti* dicarboxylate transport mutants. Cosmids, stored as a λ lysate, were introduced by transfection into *E. coli* S17.1, which was then mated *en masse* with the different *R. meliloti* mutants again. As pSUP205 is unable to replicate autonomously in *R. meliloti*, it can only survive by integrating into the genome. Since the cosmids contain *R. meliloti* DNA the integration normally occurs via crossing-over in the homologous region. Each mating mixture was plated on TY agar plus streptomycin and tetracycline to determine the frequency of mobilization of cosmids into *R. meliloti*. By additional selection on minimal medium plus streptomycin and tetracycline and with succinate as sole carbon source, cosmid clones were isolated that complemented the mutants, enabling them to utilize succinate again. By mating an RP4-derivative (pNC206) into the mutants, spontaneously excised cosmids could be mobilized back into *E. coli* S17.1, where they could be further analysed.

Fig. 1

Fig. 1. Competitive inhibition of succinate uptake by fumarate and malate in *R. meliloti* 2011. [^{14}C]Succinate uptake rates were measured for five succinate concentrations without inhibitor ($\square$), for three succinate concentrations plus 0.1 mM-fumarate ($\triangle$) and for three succinate concentrations plus 0.1 mM-malate ($\circ$). v_0 , initial velocity [$\text{nmol min}^{-1} (\text{mg protein})^{-1}$]; $[S]$, substrate concentration (nm).

Fig. 2

Fig. 2. Induction of the dicarboxylate transport system in *R. meliloti* 2011. Cells were centrifuged after growth in minimal medium plus glucose (10 mM), washed and resuspended in minimal medium plus succinate (10 mM). From this culture samples were withdrawn at intervals of 15 min and initial rates of [^{14}C]succinate uptake were measured. The succinate concentration in the uptake assay was 625 μM . Since the cells divided during the experiment, all values are corrected by the factor of increased bacterial density.

Table 2. Inhibition of [^{14}C]succinate uptake by various carbon compounds in *R. meliloti* 2011

Inhibitor	Uptake rate of [^{14}C]succinate (%)*	
	100 μM -Inhibitor	1 μM -Inhibitor
Unlabelled succinate	48	11
Fumarate	49	12
Malate	65	18
Glucose	100	98

* The [^{14}C]succinate concentration was 100 μM . The uptake rate of [^{14}C]succinate without inhibitor (= 100%) was 23 $\text{nmol min}^{-1} (\text{mg protein})^{-1}$.

RESULTS

Biochemical characterization of the C_4 -dicarboxylate transport in free-living *R. meliloti* 2011

To find out whether there is a common transport system for succinate, fumarate and malate in *R. meliloti*, inhibition studies were done with the wild-type strain 2011 (Table 2). Uptake rates of [^{14}C]succinate (100 μM) were measured without inhibitor or with the addition of unlabelled succinate, malate, fumarate or glucose at 100 μM or 1 mM. Uptake of succinate was strongly inhibited by unlabelled succinate, fumarate or malate, whereas glucose had no effect. To prove that the inhibition of succinate by fumarate and malate was competitive, succinate uptake rates at different succinate concentrations without inhibitor and with fumarate or malate as inhibitor were measured. When these data were fitted to the Michaelis-Menten equation (Lineweaver-Burk plot), the lines for the noninhibited and inhibited uptake rates met at a point close to the y-axis, indicating competitive inhibition (Fig. 1). This means that succinate, fumarate and malate are transported in *R. meliloti* by a common dicarboxylate transport system.

Initial [^{14}C]succinate uptake velocities were measured at succinate concentrations between 0.5 μM and 1.0 mM, and these values were fitted to the Michaelis-Menten equation. After linear regression calculations, a K_m of 5.3 μM and a V_{max} of 23.9 $\text{nmol (mg protein)}^{-1} \text{ min}^{-1}$ were obtained.

Table 3. Characteristics of *R. meliloti* mutants unable to grow on succinate as sole carbon source

<i>R. meliloti</i> strain	Growth on succinate, malate or fumarate	Dicarboxylate transport in		Acetylene reduction	Complementing cosmids
		Free-living cells	Symbiotic cells		
2011	++	++	++	++	
RMS11	—	—	+	+	None
RMS16	—	—	NT	+	pRmSC121
RMS17	—	—	+	+	pRmSC121
RMS24	—	—	+	+	pRmSC121
RMS118	—	—	+	+	pRmSC121
RMS420	—	—	—	—	pRmSC102/101
RMS938	—	—	—	—	pRmSC121

++, Wild-type-like activity; +, reduced activity; —, no detectable activity; NT, not tested.

To determine whether the dicarboxylate transport system is constitutively expressed or is regulated as an inducible process, an *R. meliloti* 2011 culture, grown in glucose overnight, was resuspended in succinate-containing medium and the time course of the [¹⁴C]succinate uptake rate of this culture was determined (Fig. 2). Immediately after transfer to the fresh medium succinate uptake was not detectable. It took more than 50 min for the maximal transport rate to be reached, indicating that dicarboxylate transport is an inducible process in *R. meliloti*. As a control the same culture was grown overnight in succinate. This culture showed the maximal transport rate immediately after transfer (data not shown). When cells were grown overnight in the presence of equimolar concentrations of succinate and glucose their [¹⁴C]succinate uptake rate was comparable to that of cells pregrown in succinate only (data not shown), indicating that the dicarboxylate transport system is not repressed by glucose.

To determine whether the dicarboxylate transport system is an active or passive process, the uptake rate was measured for cells exposed to different concentrations (10 µM to 2 mM) of the uncoupler DNP prior to the addition of [¹⁴C]succinate. The inhibition of succinate uptake was more than 90% at a DNP concentration of approximately 0.5 mM or more, indicating that the dicarboxylate transport system in *R. meliloti* is energy dependent and therefore represents an active process.

Isolation and characterization of Tn5-induced R. meliloti mutants defective in C₄-dicarboxylate transport

Mutants of *R. meliloti* defective in the dicarboxylate transport system were generated by random Tn5 mutagenesis. About 8 × 10³ Tn5-induced mutants were tested for their colony-forming ability on succinate minimal medium. Seven colonies were found to be unable to grow on succinate minimal medium but could form colonies on glucose minimal medium.

These seven mutants (RMS11, RMS16, RMS17, RMS24, RMS118, RMS420 and RMS938) were tested for growth in liquid medium containing different carbon sources. All the mutants failed to grow with succinate, fumarate or malate (Table 3), whereas growth with pyruvate or glutamate was similar to that of the wild-type. All seven mutants also grew at approximately wild-type rates with glucose, arabinose, fructose, sucrose, maltose, galactose or lactose as carbon source.

The succinate, malate and fumarate non-utilizing mutants were examined for their ability to take up succinate under free-living conditions. Cells were grown in minimal medium plus 10 mM-succinate and 10 mM-glucose to allow the initial growth of cells and induction of the dicarboxylate transport system. The cells were extensively washed, resuspended in minimal medium plus succinate and uptake rates were measured (Fig. 3). While the wild-type strain took up succinate, the seven mutants showed no detectable uptake. Similar results were obtained for malate (data not shown). These results indicate the absence of a functional dicarboxylate transport system in all seven mutants under free-living conditions. These mutants were therefore

Fig. 3

Fig. 4

Fig. 3. Succinate uptake of the *R. meliloti* wild-type strain 2011 (○) and the mutant strain RMS11 (●) in the free-living state. Cells were grown in minimal medium plus glucose (10 mM) and succinate (10 mM), then washed and resuspended in minimal medium plus succinate. The succinate uptake of mutants RMS16, RMS17, RMS24, RMS118, RMS420 and RMS938 was similar to that of RMS11 (data not shown).

Fig. 4. Succinate uptake by bacteroids isolated from nodules induced by *R. meliloti* wild-type and mutant strains. Uptake rates were determined immediately after bacteroid isolation. ●, Wild-type strain 2011; □, RMS11; ○, RMS17; △, RMS24; ×, RMS118; ▽, RMS420; ■, RMS938.

designated Dct^{fl-} . Revertants of all mutants (RMS938 was not tested) which were isolated on minimal medium plus succinate could also grow on malate and fumarate again.

The *R. meliloti* wild-type strain 2011 and the seven mutants were also tested for their ability to take up $[^{14}C]$ succinate and $[^{14}C]$ malate in the bacteroid state. Alfalfa seedlings were inoculated with the wild-type and also with the mutant strains. After 21 d growth, plants were harvested and bacteroids were isolated from the nodules. Uptake rates for succinate (Fig. 4) and malate (data not shown) were determined. Bacteroids of mutants RMS11, RMS17, RMS24 and RMS118 took up succinate and malate, although at only 30–50% of the wild-type rate. Bacteroids of mutants RMS420 and RMS938 did not show uptake activity with either succinate or malate (Table 3). These results suggest that the mutants RMS11, RMS17, RMS24 and RMS118 can be characterized in the symbiotic state by a reduced activity of the dicarboxylate transport system ($Dcts^{red}$), whereas the mutants RMS420 and RMS938 are defective in the dicarboxylate transport system ($Dcts^{-}$).

Further experiments revealed that the succinate and malate uptake activity of the mutants was correlated with their nitrogen-fixation activity (Table 3). When the ability of bacteroids in nodules to reduce acetylene to ethylene was measured at the harvest time (21 d), mutants RMS420 and RMS938 did not show any acetylene reduction. The nodules induced by these mutants were small and white. These two mutants are evidently Fix^{-} . All other mutants showed acetylene reduction, although at a lower rate than the wild-type (Table 3). These mutants gave rise to pink nodules of normal size. This class of mutants is therefore considered to be Fix^{red} .

Characterization of cosmid clones that complement different C_4 -dicarboxylate transport mutants

In order to isolate DNA fragments carrying *dct* genes, the mutants described so far were each used as recipients for cosmids of an *R. meliloti* 2011 clone bank which were introduced *en masse* into the mutants. Tc^r transconjugants were obtained from all mating mixtures at a frequency of 10^{-4} per recipient after 4–5 d growth. Two cosmids, designated pRmSC101 and pRmSC102, restored growth of the Fix^{-} mutant RMS420 on succinate, fumarate and malate (Table 3). Plant

Fig. 5. Complementation analysis of *R. meliloti* mutant RMS420 by partially deleted inserts of pRmSC102. A restriction map of the *Eco*RI insert of cosmid pRmSC102 is shown. By partial *Hind*III digestion, five different deletion cosmids (pRmSC102H1, -H2, -H3, -H4, -H5) were constructed. The locations of the individual deletions are indicated. The ability of these deletion cosmids to complement mutant RMS420 was tested (a) by growth on succinate, malate or fumarate and (b) by an assay to form effective nodules. The *Eco*RI-*Hind*III fragment indicated at the bottom is evidently necessary for complementation of mutant RMS420 whereas the indicated 15.3 kb *Eco*RI fragment carries the Tn5 transposon in mutant RMS420 (see Fig. 6).

tests of RMS420(pRmSC101) and RMS420(pRmSC102) indicated that the effective symbiotic nitrogen-fixation phenotype was also restored to the wild-type level. Both pRmSC101 and pRmSC102 were unable to complement any of the other mutants described in Table 3.

A third cosmid clone, pRmSC121, was isolated from the gene bank, which complemented most of the other dicarboxylate transport mutants. Cosmid pRmSC121 restored growth on succinate, malate and fumarate for mutants RMS16, RMS17 and RMS24, RMS118 and RMS938 (Table 3). The frequency of complementation for the different mutants differed widely and has to be investigated by a detailed analysis in future. Interestingly, cosmid pRmSC121 did not complement mutant RMS420 complemented by cosmids pRmSC101 and pRmSC102, which means that the two groups of isolated cosmids do not cross-complement the mutants under investigation.

To analyse the recombinant cosmids, pRmSC101 and pRmSC102 cosmid DNA was isolated on a large scale from *E. coli* K802 and analysed by *Eco*RI and *Hind*III restriction endonucleases. The vector pSUP205 contains single *Eco*RI and *Hind*III cleavage sites, and restriction with *Eco*RI separates the vector from the insert fragments in the cosmids. The restriction pattern of the two cosmids revealed the presence of ten *Eco*RI and six *Hind*III fragments in pRmSC101 with a total size of approximately 43 kb, and thirteen *Eco*RI and seven *Hind*III fragments in pRmSC102 with a total size of 47 kb. Cosmid pRmSC102 contained all the *Eco*RI fragments of pRmSC101 and three extra *Eco*RI fragments extending pRmSC101 at the right-hand end. The larger cosmid pRmSC102 was used for further analysis.

*Hind*III cleaved pRmSC102 into seven fragments. The order of the *Hind*III fragments within pRmSC102 was established (Fig. 5) by partial digestion and ligation followed by transformation of *E. coli* S17.1 and analysis of plasmid DNA from the transformants. The ordering was based on the frequency of the individual *Hind*III fragments present or absent in the religated plasmid DNA molecules. A number of hybrid plasmids containing deletions of various *Hind*III fragments were generated in this experiment; these plasmids are shown in Fig. 5. The *Hind*III fragments flanking the vector DNA were verified by hybridization of the *Hind*III-restricted pRmSC102 DNA with ³²P-labelled vector pSUP205 DNA. The relative positions of the

Fig. 6. Southern hybridization analysis to localize the site of Tn5 insertion in mutant RMS420. DNA of cosmid pRmSC102 (lanes 1), and total DNA of wild-type 2011 (lanes 2) and mutant RMS420 (lanes 3) was restricted with *Eco*RI, separated by agarose gel electrophoresis, transferred to nitrocellulose filters and probed with ^{32} P-labelled pRmSC102 (a) or pSUP5011 (containing Tn5) (b). The 8.7 kb fragment represents vector DNA.

restriction sites for *Eco*RI and *Bam*HI were identified by single and double digestions of purified pRmSC102 DNA, and the various restriction endonuclease cleavage sites are shown in Fig. 5.

Further proof for the location of the mutation in mutant RMS420, presumably due to Tn5 insertion, was obtained by complementation analysis involving various deletion derivatives of pRmSC102 (Fig. 5). Hybrid cosmids containing deletions were mobilized from *E. coli* S17.1 by conjugation into mutant RMS420. $\text{Sm}^r \text{Tc}^r$ transconjugants were isolated and tested for growth on minimal medium containing succinate. Hybrid cosmids pRmSC102H1 and pRmSC102H5 were unable to complement for growth on succinate. The remaining three pRmSC102H cosmids were able to complement mutant RMS420, indicating that the complementing region was located in the 6.9 kb *Hind*III fragment and perhaps partly in the adjacent large *Eco*RI–*Hind*III fragment (Fig. 5). The hybrid cosmids containing these two fragments were also able to restore the nitrogen-fixation phenotype to the wild-type level. The restriction analysis of pRmSC102 indicated that the 6.9 kb *Hind*III fragment was present within the large 15 kb *Eco*RI fragment.

Mapping of the Tn5 insertion site of mutant RMS420

To localize the site of insertion of transposon Tn5 in mutant RMS420, Southern hybridization experiments were done. Total DNA was isolated from the wild-type strain 2011 and mutant RMS420, restricted with *Eco*RI, separated by agarose gel electrophoresis (0.7%) and transferred to nitrocellulose filters. The nitrocellulose blots were hybridized to ^{32} P-labelled pRmSC102 DNA. Tn5 contains no *Eco*RI recognition sites. If the site of the Tn5 insertion is in the region of the genome corresponding to the complementing cosmid pRmSC102, then the altered fragment should hybridize to the ^{32} P-labelled plasmid. Thus, while in the wild-type the pattern of

hybridization bands should be like that of pRmSC102 itself, in the mutant RMS420 the *Eco*RI fragment containing Tn5 should alter by the addition of the size of Tn5 (5.75 kb, Reznikoff, 1982). The autoradiogram shown in Fig. 6 revealed the alteration of the largest *Eco*RI fragment (corresponding to a size of approximately 20 kb) in the mutant but not in the wild-type, indicating the presence of Tn5 in this fragment. The largest fragment in pRmSC102 had a size of approximately 15 kb. A duplicate nitrocellulose blot was hybridized to ³²P-labelled pSUP5011 DNA (containing Tn5). While there was no hybridization to the wild-type DNA, a fragment of approximately 20 kb in the mutant DNA did hybridize (Fig. 6), consistent with the previous indication that Tn5 was located in the 15 kb *Eco*RI fragment. When the two autoradiograms were superimposed, the two bands were present in the same position.

DISCUSSION

The C₄-dicarboxylate transport system of free-living *R. meliloti* 2011 was investigated by physiological and genetic studies. It was shown that succinate uptake is strongly inhibited by fumarate and malate and that this inhibition is competitive. These results were confirmed by a mutational analysis. Tn5-induced mutants which could not grow on succinate, also did not grow on fumarate or malate but did grow on other carboxylic acids (pyruvate and glutamate). Revertants of the mutants grew on all three dicarboxylic acids again. Succinate uptake and malate uptake in these mutants were absent. All these results give evidence for a functional C₄-dicarboxylic acid transport system in free-living *R. meliloti*. Dicarboxylate transport systems in free-living cells have been shown in *E. coli* (Kay & Kornberg, 1971; Lo *et al.*, 1972), *Bacillus subtilis* (Ghei & Kay, 1973), *Azotobacter vinelandii* (Reuser & Postma, 1973), *Pseudomonas putida* (Dubler *et al.*, 1974), *Salmonella typhimurium* (Kay & Cameron, 1978), '*R. trifolii*' (Ronson *et al.*, 1981), *R. leguminosarum* (Glenn *et al.*, 1980; Finan *et al.*, 1981) and *Bradyrhizobium japonicum* (McAllister & Lepo, 1983; San Francisco & Jacobson, 1985). Succinate-uptake-deficient mutants of *R. meliloti* were recently described by Bolton *et al.* (1986).

The dicarboxylate transport system in free-living *R. meliloti* is inducible by succinate within more than 50 min. The system is not induced if glucose is the only carbon source, which was also reported for fast-growing cowpea rhizobia (Saroso *et al.*, 1984). In contrast, an equimolar mixture of succinate and glucose keeps the system induced, indicating that glucose does not suppress the dicarboxylate transport system in *R. meliloti*. Partial suppression by glucose was reported for '*R. trifolii*' by Ronson *et al.* (1981). Inducible dicarboxylate transport systems have been found in '*R. trifolii*' (Ronson *et al.*, 1981), *R. leguminosarum* (Finan *et al.*, 1981) and *B. japonicum* (McAllister & Lepo, 1983), while constitutive dicarboxylate transport systems were reported for *R. leguminosarum* (Glenn *et al.*, 1980) and two *Bradyrhizobium* species (San Francisco & Jacobson, 1985). The dicarboxylate transport system in free-living *R. meliloti* is energy dependent, as judged by the effects of DNP. More information is required about the precise link of the dicarboxylate transport system to an energy source in *Rhizobium*.

Seven mutants lacking the dicarboxylate transport system under free-living conditions (Dctf⁻) were isolated. After differentiation of free-living cells into bacteroids in alfalfa nodules, only the two mutants RMS420 and RMS938 were still dicarboxylate transport deficient (Dcts⁻), whereas bacteroids of four other mutants (RMS11, RMS17, RMS24 and RMS118) showed active dicarboxylate transport but at a reduced rate (Dcts^{red}). We do not know the nature of the differences between these two systems. However, it may be that there exists a complex regulation responsible for the expression of the relevant genes under free-living and symbiotic conditions. Dcts⁻ mutants RMS420 and RMS938 were Fix⁻, whereas Dcts^{red} mutants RMS11, RMS17, RMS24 and RMS118 were also Fix^{red}. This correlation suggests that the C₄-dicarboxylate transport in bacteroids is limiting the nitrogen-fixation capacity of nodules. In addition, one can speculate that it may be possible to enhance symbiotic nitrogen fixation by improving dicarboxylate transport in bacteroids (see Bolton *et al.*, 1986).

Other authors have stated that a functional dicarboxylate transport system is not necessary for nodulation (Ronson *et al.*, 1981; Finan *et al.*, 1983; Arwas *et al.*, 1985). The same result was obtained in this investigation. Nodules induced by Dcts⁻ mutants were smaller than wild-type

nodules and their white colour indicated the absence of leghaemoglobin. The nodules were colonized by bacteroids and the typical zonation could be detected by light microscopy (D. Kapp, personal communication).

Genetic complementation of the Fix^- mutant RMS420 with the cosmids pRmSC101 or pRmSC102 always resulted in growth on succinate and in the restoration of nitrogen-fixation capacity to the wild-type level. We know from our genetic studies that the complementing information is located on a 19.4 kb *EcoRI*-*HindIII* fragment containing a 6.9 kb *HindIII* fragment. Hybridization data suggest that the Tn5 transposon of mutant RMS420 is located on a 15.3 kb *EcoRI* fragment which includes the 6.9 kb *HindIII* fragment. Both pRmSC101 and pRmSC102 were unable to complement the other mutants. A third cosmid, pRmSC121, showed a completely different complementation pattern, complementing the Fix^- mutant RMS938 and most of the Fix^{red} mutants. Thus it seems that this cosmid carries most of the genetic information coding for C_4 -dicarboxylate transport in *R. meliloti*.

Our results indicate the importance of the C_4 -dicarboxylate transport system for symbiotic nitrogen fixation in *R. meliloti*. Isolation of two unrelated cosmids complementing most of the mutants provides material for a detailed molecular analysis in the future.

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