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Symbiotic Properties of C_4-Dicarboxylic Acid Transport Mutants of *Rhizobium leguminosarum*

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The transport of succinate was studied in bacteroids of an effective, streptomycin-resistant strain (GF160) of *Rhizobium leguminosarum*. High levels of succinate transport occurred, and the kinetics, specificity, and sensitivity to metabolic inhibitors were similar to those previously described for free-living cells. The symbiotic properties of two transposon (Tn*5*)-mediated C_4-dicarboxylate transport mutants (strains GF31 and GF252) were determined. Strain GF31 formed ineffective nodules, and bacteroids from these nodules showed no succinate transport activity. Strain GF252 formed partially effective nodules, and bacteroids from these nodules showed about 50% of the succinate transport activity of the parent bacteroids. Another dicarboxylic acid transport mutant (Dct^-), strain GFS5, isolated after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis, formed ineffective nodules. The ability to form ineffective nodules in strains GF31 and GFS5 was shown to correlate with the Dct^- phenotype. The data indicate that the presence of a functional C_4-dicarboxylic acid transport system is essential for N_2 fixation to occur in pea nodules.

The supply of photosynthate limits N_2 fixation by nodulated leguminous plants (13). There is now increasing evidence that C_4-dicarboxylic acids produced by the plant are utilized by bacteroids in N_2-fixing root nodules (2, 10, 27, 28). Recently, we reported that free-living cells of *Rhizobium leguminosarum* GF160 possess a C_4-dicarboxylic acid transport system which is inducible and mediates the active transport of succinate, fumarate, and malate into the cell (9).

In this study, C_4-dicarboxylic acid transport mutants were used to examine the importance of bacteroid C_4-dicarboxylic acid transport in pea root nodules. The evidence presented indicates that bacteroids of *R. leguminosarum* possess a C_4-dicarboxylic acid transport system similar to that found in free-living cells and that the presence of a functional C_4-dicarboxylate transport system in bacteroids is essential for N_2 fixation to occur in pea nodules.

(This work was presented in part at the Annual Meeting of the Canadian Society of Microbiologists, Ottawa, 1981.)

MATERIALS AND METHODS

Bacterial strains, phage, and media. Table 1 lists the bacterial strains and phage used in this study.

TY medium was prepared as described previously by Beringer (3). Preparation of TY-succinate, MOPS (morpholinepropanesulfonic acid) salts, (MS), MYC medium, and the general culture conditions were as previously described (9). The plant nutrient solution (SPNS) was prepared as described previously by Schwinghamer (29). Each liter contained the following: KCl, 0.5 g; KH_2PO_4, 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaSO_4 \cdot 2H_2O$, 0.2 g; and trace elements (9). To avoid precipitation, we autoclaved KH_2PO_4 (pH adjusted to 7.2 with NaOH) separately from the rest of the solution.

Transduction. Transduction with UV-irradiated phage RL38 was performed as described previously by Buchanan-Wollaston (5). Phage suspensions were maintained over chloroform. The absence of bacteria in the phage lysates was confirmed for each experiment by spreading the phage suspensions on MYC medium. Kanamycin-resistant transductants were selected by spreading the transduction mixture on MYC medium containing kanamycin (20 μg/ml). Transductants of strains GF31 and GFS5 which could grow on succinate were selected by washing the transduction mixture with 0.9% saline and plating on succinate (15 mM)–MS.

Plant nodulation tests. Pea seeds (*Pisum sativum*, variety Trapper) were surface sterilized by immersion in concentrated H_2SO_4 for 20 min. The seeds were then washed thoroughly with at least 10 changes of sterile water over a 2-h period, spread on water-agar medium (1% [wt/vol] Difco agar), and incubated in the dark for 2 days at room temperature.

Plants were grown aseptically in a vermiculite-quartz sand mixture (1:1), using Leonard jar assemblies (16). About 2 or 3 days after the seedlings were placed in the jars, 100 ml of SPNS containing approximately 10^8 bacteria per ml was added to each jar.

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TABLE 1. Bacterial strains and phage

Strain or phage	Relevant phenotype[a]	Origin
R. leguminosarum		
GF160	Str^r	Finan, Wood, and Jordan (9)
GF252	Str^r Dct^- Km^r	Tn*5* insertion mutant of GF160
GF31	Str^r Dct^- Km^r	Tn*5* insertion mutant of GF160
GF131	Str^r Dct^- Km^r	Transductant of GF160 from GF31
GF132	Str^r Dct^- Km^r	Transductant of GF160 from GF31
GF310	Str^r	Transductant of GF31 from GF160
GF312	Str^r	Transductant of GF31 from GF160
GF33	Str^r Dct^- Km^r	Tn*5* insertion mutant of GF160
GF351	Str^r Dct^- Km^r	Tn*5* insertion mutant of GF160
GFS5	Str^r Dct^-	NTG-induced mutant of GF160
GFS51	Str^r	Transductant of GFS5 from GF160
GFS18	Str^r Sdh^-	NTG-induced mutant of GF160
Phage (RL38)		Generalized transducing phage (5)

[a] Str^r, Streptomycin resistance; Sdh^-, reduction in succinate dehydrogenase activity; Dct^-, dicarboxylic acid transport defective; Km^r, kanamycin resistance.

Plants were grown under warm, white fluorescent light supplemented with either incandescent bulbs or Sylvania Grolux tubes with alternating light and dark periods of 16 and 8 h, respectively. The light intensity was approximately 20,000 lx. Plants were grown in the light period at a temperature of 20 to 23°C and in the dark period at a temperature of 15°C. After a total of 300 ml of SPNS was added to each jar, the plants were additionally watered when necessary by adding sterile distilled water.

Plant dry weight determinations. Plant tops from each jar were cut at the stem-root juncture and placed in an oven at 80°C for 48 h. The plant tops were then weighed, and an additional 48 h of drying did not change the dry weight values obtained.

Bacteroid isolation. The procedure used was modified from the methods used by Planque et al. (24) and Laane et al. (15). MMS buffer (pH 7) contained the following: MOPS, 40 mM; KOH, 20 mM; $MgSO_4$, 2 mM; and sucrose, 0.3 M. The root systems of 10 to 16 pea plants inoculated and grown as described above were washed thoroughly with water. The nodules were then removed over a time period of about 15 min and placed in a beaker at 4°C. Attempts were made to minimize the inclusion of root tissue. The nodules were then placed in a mortar with 15 ml of MMS containing 4% polyvinylpyrrolidone (BDH; molecular weight, ca. 40,000) and vigorously homogenized with a pestle. The resulting homogenate was passed through eight layers of cheesecloth, and the resulting filtrate was centrifuged at 500 × *g* for 5 min to remove plant cell fractions and other debris. The clarified homogenate then was centrifuged at 5,000 × *g* for 10 min, and the supernatant was discarded. The pellet containing the bacteroids was suspended to 20 ml with MMS containing fatty-acid-free bovine serum albumin (3 mg/ml) and again centrifuged at 5,000 × *g* for 10 min. The supernatant was discarded, and the pellet was washed twice with 20 ml of MMS by centrifuging at 5,000 × *g* for 5 min. The final pellet was suspended in MMS to give about 4 mg of bacteroid protein per ml. This suspension appeared to contain a pure preparation of bacteroids when examined by phase-contrast microscopy. The preparations from ineffective nodules contained many pleiomorphic cells which were similar to bacteroids from effective nodules. They are also described here as bacteroids.

Transport assays. Transport activities of bacteroids were determined immediately upon completion of the bacteroid isolation procedure. The method used was as described previously for transport assays in free-living cells (9). All assays were performed in triplicate on bacteroids preincubated in MMS for 5 min at 30°C. After the addition of the radioactive substrate, samples were withdrawn at time intervals, rapidly filtered through membrane filters (0.45-μM pore size; Millipore or Gelman), and immediately washed with 10 to 15 ml of MMS which had been prewarmed to room temperature. Initial rate determinations were derived from measurements of radioactivity accumulated in 15 and 30 s. Competition experiments were performed as previously described (9). In experiments on the effects of metabolic inhibitors on transport, the cells were preincubated for 9 min with the inhibitor at 30°C before the addition of ^{14}C substrate. Kinetic data were analyzed by nonlinear least-squares regression analysis (9).

Reisolation of bacteria from nodules. Nodules attached to a piece of root were washed twice with sterile distilled water and surface sterilized by immersion in a solution containing 1% (wt/vol) available chlorine for 8 min. The nodules were washed in TY medium, squashed in a drop of the same medium, and streaked directly onto media as described below.

Acetylene reduction. Pieces of root with attached nodules were cut from the plants and immediately placed in 63-ml serum vials. The vials were immediately capped (serum stopper), and 6.3 ml of acetylene was injected into each. The excess gas was released from the vials, and 0.5-ml samples were removed at 15- and 30-min intervals. The samples were analyzed for ethylene by gas chromatography as previously described (4). Under these conditions, the acetylene-reducing activity of the root nodules was found to be linear with respect to time for at least the first 30 min.

Electron microscopy. Root nodules were removed

TABLE 2. Succinate, malate, and D-lactate transport in mutant strains of *R. leguminosarum* GF160

Strain	Transport (nmol/min per mg of protein)[a]			
	Succinate*	Succinate‡	Malate‡	D-Lactate‡
GF160	18	83	50	28
GF252	<0.5	<1	<1	31
GF31	<0.5	<1	<1	30
GF33	<0.5	28	17	31
GF351	<0.5	13	10	30
GFS5	ND[b]	<1	<1	32

[a] Bacteria were grown in TY-succinate (*) or oxaloacetate-MS (‡) medium. Succinate, malate, and D-lactate uptake was measured at substrate concentrations of 25, 20, and 12.5 μM, respectively. The assays were performed on cells preincubated in the presence of glucose (15 mM). The data shown are the means of triplicate assays.

[b] ND, Not determined.

from plants 20 days after inoculation and washed in 0.025 M potassium phosphate buffer (pH 6.8). Slices from the central area of effective nodules and whole ineffective nodules were fixed in 2.5% (vol/vol) glutaraldehyde in 0.025 M potassium phosphate buffer (pH 6.8) at room temperature. Unless otherwise stated, all subsequent steps were carried out at 4°C. The tissue was washed with the phosphate buffer for 4 h and postfixed in 1% (wt/vol) osmium tetroxide in phosphate buffer for 2 h. After washing in buffer overnight, the tissue was suspended in 2% (wt/vol) aqueous uranyl acetate for 2 h, washed with distilled water for 2 h, dehydrated in an ascending series of acetone concentrations, and kept in 100% acetone for 30 min. The tissue then was transferred to a 1:1 (vol/vol) mixture of acetone plus Spurr's low-viscosity embedding medium (32) in a glass petri dish. The acetone was allowed to evaporate overnight at room temperature, and the tissue was then transferred to fresh Spurr's embedding medium and baked at 70°C for 12 h. Thin sections were cut with a Sorvall Ultra microtome, transferred to Formvar-coated grids, and stained with 1% (wt/vol) uranyl acetate (pH 4.5), followed by lead citrate (26), before viewing with a Phillips 300 transmission electron microscope.

Protein determination. Protein was determined by the method of Lowry et al. (18), using bovine serum albumin as the standard. Whole cells and bacteroids were solubilized by boiling in 1 N NaOH before assay.

RESULTS

C_4-dicarboxylic acid transport mutants. The C_4-dicarboxylate transport mutants GF252, GF31, GF33, and GD351 were isolated after Tn*5* mutagenesis, whereas strain GFS5 was isolated after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis as previously described (9). These mutants were unable to grow on succinate, fumarate, or malate, although they all grew normally on glucose, ribose, pyruvate, citrate, or oxaloacetate as the sole carbon source. When these strains were grown in TY-succinate medium, all of the mutants examined showed negligible succinate transport activity (Table 2). Since oxaloacetate induced the succinate transport system in GF160 (9), the effect of growing the mutants on this compound as sole carbon source was investigated (Table 2). Strains GF33 and GF351 showed significant succinate and malate transport activities under these conditions, whereas strains GF252, GF31, and GFS5 were succinate and malate transport negative. Because of the leaky nature of the transport defects in strains GF33 and GF351, their symbiotic properties were not examined.

The mutant strains were not generally deficient in transport, as all of the strains had similar D-lactate transport activities (Table 2). Furthermore, succinate (at 8- or 40-fold excess) did not inhibit D-lactate transport in strain GF160, GF252, or GF31 (T. Finan, Ph.D. thesis, University of Guelph, Guelph, Ontario, Canada, 1981). These data imply that, in contrast to the *Escherichia coli* system (17), D-lactate transport in *R. leguminosarum* does not require a catalytic component common to the C_4-dicarboxylate transport system. Evidence that the Dct^- phenotype of strain GF252 is due to a single mutation has been presented (9). Of 50 Dct^+ revertants isolated on succinate-MS medium, 47 were kanamycin sensitive. This indicates that the Dct^- mutation in GF252 is closely linked to a single Tn*5* insertion. Whereas Dct^+ revertants of GF252 were isolated at a frequency of about 10^{-7} on succinate-MS medium, the Dct^+ reversion frequencies for the transport mutants GF31 and GFS5 were determined to be less than 10^{-9}, and no spontaneous Dct^+ revertants of these strains were isolated. However, through the use of a recently isolated generalized transducing phage (RL38) of *R. leguminosarum* (5), it was possible to transduce the succinate transport mutation from GF31 to GF160. Selection was made for kanamycin-resistant transductants as described above. The frequency of kanamycin resistance transfer was approximately 5×10^{-6} per recipient, whereas the spontaneous frequency of kanamycin resistance was appoximately 10^{-9}. In one experiment, 95 of 96 kanamycin-resistant transductants screened were unable to

TABLE 3. Symbiotic properties of *R. leguminosarum* strains[a]

Inoculum	Dry wt of plant tops (g/3 plants)		Nodulation[b]
	Expt 1	Expt 2	
GF160	5.0 ± 0.8 (4)	3.1 ± 0.3 (4)	+
GF252	2.4 ± 0.3 (4)	1.9 ± 0.1 (4)	+
GF31	0.4 ± 0.1 (4)	0.4 ± 0.1 (4)	+
GF131	0.5 ± 0.1 (3)	ND[c]	+
GF132	0.5 ± 0.1 (3)	ND	+
GF310	ND	2.7 ± 0.3 (3)	+
GF312	ND	3.7 ± 0.5 (3)	+
GFS5	0.4 ± 0.1 (4)	0.4 ± 0.1 (4)	+
GFS51	ND	3.2 ± 0.4 (4)	+
GFS18	0.6 ± 0.1 (4)	ND	+
Uninoculated	0.3 ± 0.1 (4)	0.4 ± 0.1	−

[a] Plants were grown in Leonard jars (three plants per jar) and inoculated as described in the text. Thirty-seven (experiment 1) and 34 (experiment 2) days after inoculation, the plant tops for each jar were pooled for dry weight determinations. Dry weight values are expressed as the mean ± the standard deviation. The number of jars used for each strain is shown in parentheses.

[b] +, Root nodules present; −, root nodules absent.

[c] ND, Not determined.

grow on succinate, fumarate, or malate as the sole carbon source. This suggests that kanamycin resistance (Tn*5*) and the Dct$^-$ phenotype are very closely linked in strain GF31. Two of the above transductants, designated GF131 and GF132, were retained for additional studies.

Transductants of GF31 which were capable of growth on succinate were isolated by transducing GF31 with phage grown on GF160 and selecting for transductants which could grow on succinate-MS. These arose at a frequency of approximately 5×10^{-6} per recipient plated. Each of the 50 transductants tested grew on succinate, malate, and fumarate as sole carbon sources. Two of these transductants were subsequently streaked on succinate-MS, and stock cultures were made from single-colony isolates of each. These were designated GF310 and GF312, and they were sensitive to kanamycin (20 μg/ml). Dct$^+$ transductants of GFS5 were similarly isolated. One of these transductants, designated GFS51, was purified and used in further studies.

Symbiotic properties of Dct$^-$ mutants. The effectiveness of the rhizobial strains was examined by comparing the dry weights of uninoculated and inoculated plants grown under nitrogen-deficient conditions (31). In some experiments, the nitrogen-fixing ability of nodules was also determined by the acetylene-reduction technique. Ineffective strains were defined as those in which the dry weights of plants inoculated with the strains were not significantly different from those of uninoculated controls. The dry weights of plants inoculated with a partially effective strain were intermediate between those of the parent strain GF160 and the uninoculated controls. Table 3 shows that of the Dct$^-$ strains examined, strain GF252 formed partially effective nodules whereas strains GF31 and GFS5 formed ineffective nodules.

If the ineffectiveness of strains GF31 and GFS5 was due to their Dct$^-$ phenotype, then the restoration of a Dct$^+$ phenotype in these strains should also result in the restoration of effectiveness. Strains GF310 and GF312 (Dct$^+$ transductants of GF31) and strain GFS51 (a Dct$^+$ transductant of GFS5) formed effective nodules (Table 3). Also, if ineffectiveness was due to the Dct$^-$ mutation, then the transfer of this mutation to the effective parent strain GF160 should result in the transfer of the ineffective phenotype. Two such Dct$^-$ transductants, strains GF131 and GF132, were examined, and both strains formed ineffective nodules (Table 3). These data therefore showed that there was a strong correlation between the Dct$^-$ phenotype and ineffectiveness in strains GF31 and GFS5. The partially effective phenotype of the other Dct$^-$ mutant examined, strain GF252, initially suggested that the above correlation was not absolute (see below).

If C_4-dicarboxylic acids were utilized by bacteroids during N_2 fixation, then an active tricarboxcylic acid cycle in bacteroids should be required for N_2 fixation to occur in pea nodules. The symbiotic properties of a succinate dehydrogenase-deficient mutant strain GFS18 (9) were therefore examined, and this strain formed ineffective nodules (Table 2). Revertants of this mutant could not be isolated (9), and therefore, this result should be viewed with caution. However, the ineffective phenotype of strain GFS18 is consistent with that found by Duncan and Fraenkel (7) for an α-ketoglutarate dehydrogenase mutant of *Rhizobium meliloti*.

It was important to confirm that the nodules

which formed on the roots of plants after inoculation were in fact due to infection by the strain used in the inoculum, and not to contaminants or revertants. Consequently, a total of 24 nodules from plants inoculated with strain GF252 were examined for the presence of bacteria which could grow on succinate minimal medium, and no such bacteria were detected. Bacteria from all 24 nodules grew readily on glucose-MS, showing that the inability of bacteria from GF252 nodules to grow on succinate-MS was not due to an overall inability of bacteria from these nodules to grow on minimal medium. For all of the other strains examined in Table 3, the bacteria in the nodules had the same phenotype as the strain used in the inoculum. These data therefore eliminate the possibility that the symbiotic properties of the mutant strains might be due to nodule formation by revertants of the particular mutant used for inoculation. Also, nodules were never found on the roots of uninoculated control plants, which would suggest that contamination with *R. leguminosarum* via the air or contamination of one Leonard jar with bacteria from another occurred very rarely.

Nodule structure. Macroscopic examination of roots from plants inoculated with strains GF160, GF310, GF312, GFS51, and GF311 revealed the large pink nodules typical of effective nodulation. The nodules present on the roots of plants inoculated with strains GF31, GF131, GF132, and GFS5 were small and white, the characteristics typical of ineffective nodules. Although the nodules formed by strain GFS18 were also white, they were longer than the ineffective nodules formed by strain GF31. Root nodules formed by strain GF252 were pink in color and appeared to be slightly smaller than the effective nodules of strain GF160.

Thin sections of root nodules from plants inoculated with strains GF160 and GF31 were examined by electron microscopy (Fig. 1). The bacteroids present in the host cells of the ineffective nodules induced by strain GF31 appeared to be very similar to those present in strain GF160-induced effective nodules. In both cases, the bacteroids, which occupied most regions of the cytoplasm of the host cells, were surrounded by the peribacteroid membrane and were pleomorphic. However, infected cells from the ineffective nodules contained membrane-enclosed regions with up to 10 rod-shaped bacteria. The possibility that these structures were unwalled droplets (21) could not be excluded, but in some host cells up to four such structures were observed. In infected cells of ineffective nodules, premature disintegration of the bacteroids did not appear to occur.

Succinate transport in bacteroids. Initial experiments showed that bacteroids from nodules of plants inoculated with GF160 took up succinate and malate rapidly and as a linear function of time for at least 2 min (Fig. 2). In all subsequent experiments, 15- and 30-s time points were therefore used for initial rate determination.

Since free-living cells of strains GF252 and GF31 were unable to transport succinate, it was important to determine if bacteroids of these strains were also succinate transport negative. Therefore, bacteroids from nodules of plants grown for 24 days after inoculation with strain GF160, GF252, or GF31 were examined for succinate transport activity. At a succinate concentration of 50 μM, we found that the initial rate of succinate transport was 20, 11.5, or 0.5 nmol/min per mg of protein, respectively. These data showed that bacteroids of GF252 could transport succinate, whereas bacteroids of GF31 could not. In view of the proposed importance of the C_4-dicarboxylic acids as energy sources for bacteroid N_2 fixation, the data presented an obvious explanation for the difference in the symbiotic phenotypes of strains GF252 and GF31. That is, strain GF31 formed ineffective nodules because GF31 bacteroids were unable to transport succinate, whereas GF252 formed partially effective nodules because GF252 bacteroids transported succinate at a lower rate than bacteroids of the effective parent strain, GF160.

Table 4 presents the data obtained from a separate experiment in which plant top dry weight, nodule acetylene reduction, and bacteroid succinate transport values were obtained for plants 16, 23, and 31 days after inoculation with strain GF160 or GF252. Strains GFS18 and GF31 were also included in this experiment, and the data show that whereas bacteroids from GF31-induced ineffective nodules did not transport succinate, bacteroids from GFS18-induced ineffective nodules showed substantial succinate transport activity. Since GFS18 bacteroids were purified from ineffective nodules and showed transport activity, the lack of succinate transport activity of GF31 bacteroids is not solely due to their purification from ineffective nodules. The data also show that there was no obvious relationship between the bacteroid succinate transport rates and the acetylene reduction activities. This was not unexpected as the acetylene reduction rates were a measure of the nitrogenase activities of the N_2-fixing bacteroids only, averaged over 15 or 30 min, whereas the initial rates of succinate transport were determined for the entire bacteroid popultion. The succinate transport rates of GF252 bacteroids were consistently lower than the rates in GF160 bacteroids, but the difference in their transport activities appeared to decrease as the nodules aged. Also, maximum bacteroid succinate trans-

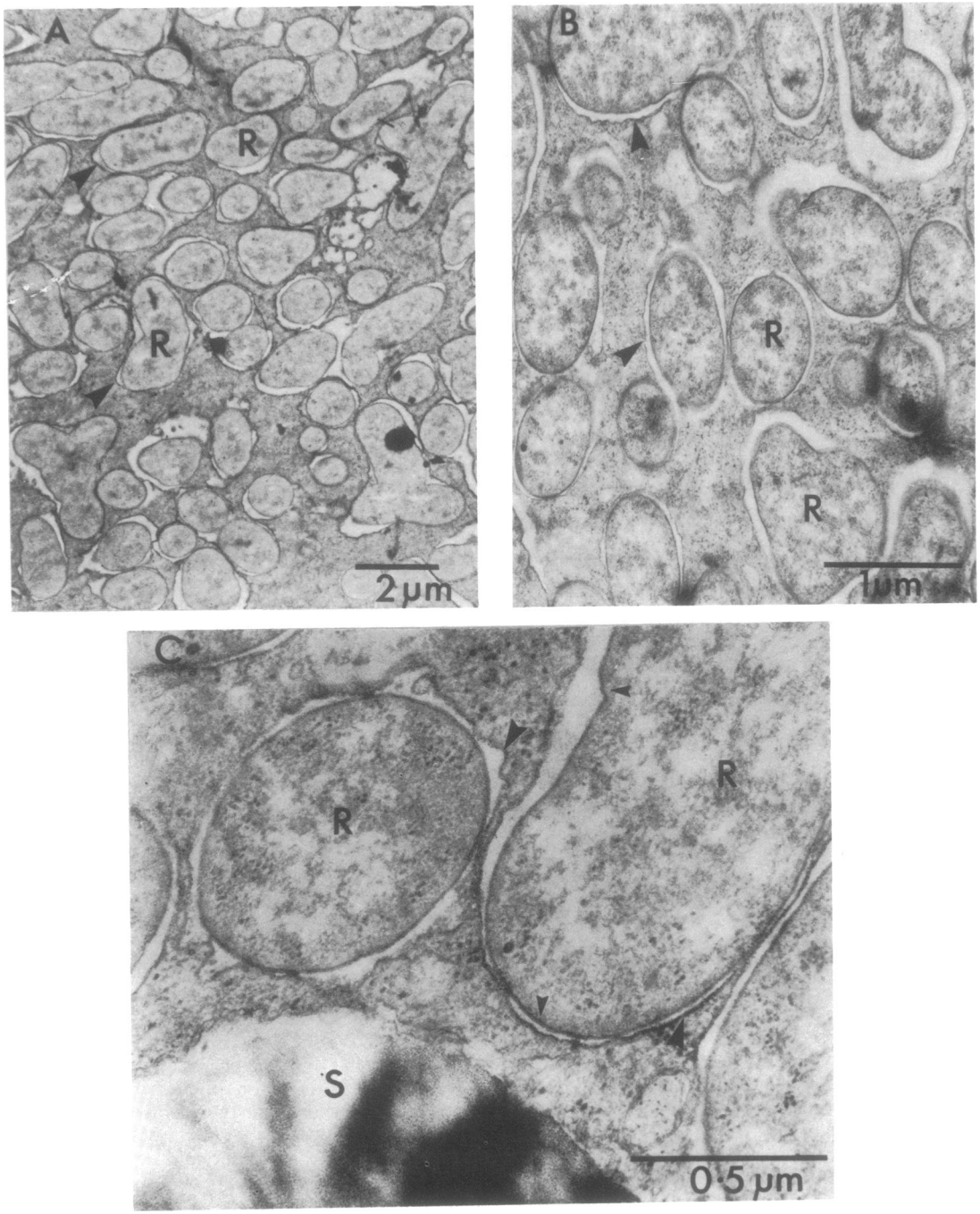

FIG. 1. (A) Electron micrograph showing part of an infected cell from a nodule formed by strain GF160; (B, C) electron micrographs showing parts of infected cells from a nodule formed by GF31. Note the bacteroids (R), the bacteroid cell envelope (small arrowheads), the peribacteroid membrane (large arrowheads), and the starch granule (S).

port activity appeared to occur approximately 23 days after inoculation. When examined, the relative malate transport activity corresponded with the succinate transport activity, suggesting that these two C_4-dicarboxylic acids are transported via a common system in bacteroids.

Kinetics of succinate transport. To determine if succinate transport in bacteroids of *R. legumin-*

osarum is a carrier-mediated process, we examined the kinetic properties of the transport system in bacteroids of GF160. Bacteroids isolated from nodules on plants inoculated with strain GF252 were also examined to determine if the kinetic properties and specificity of the succinate transport system of GF252 bacteroids were similar to those of GF160 bacteroids. The initial rates of succinate transport in bacteroids of both these strains were determined at substrate concentrations from 1.1 to 22 μM. Transport was a saturable function of substrate concentration (Fig. 3). When the data were fit to the Michaelis-Menten equation [$v = (V_{max}[S])/(K_m + [S])$], the K_m and V_{max} values obtained for strains GF160 and GF252 were: K_m = 1.5 ± 0.3 and 2.5 ± 0.3 μM and V_{max} = 22.9 ± 1.0 and 9.2 ± 0.3 nmol/min per mg of protein, respectively.

Specificity of succinate transport. The specificities of the succinate transport activities in bacteroids of GF160 and GF252 were very similar (Table 5). 2-Butylmalonate had no effect on the bacteroid transport activity, whereas phenylsuccinate inhibited slightly. Table 5 shows that the phosphate analog sodium arsenate had little effect on succinate transport in bacteroids of GF160, whereas the uncouplers 2,4-dinitrophenol (DNP) and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) caused a large reduction in transport activity. These data were similar to those obtained for the free-living bacteria (9; T. Finan Ph.D. thesis).

Muecke and Wiskich (19) have shown that mitochondria isolated from soybean nodules could oxidize succinate and malate. As mitochondria are known to transport succinate rapidly (ca. 100 nmol/min per mg of protein at 20°C [25]), it was important to determine if any of the succinate transport activity detected in the bacteroid preparations was due to the presence of contaminating mitochondria. The bacteroid-containing fraction used in this study was obtained after two 10-min centrifugations at 5,000 × *g* and was washed twice by centrifugation at 5,000 × *g* for 5 min. As centrifugation at 10,000 × *g* for 10 to 15 min is required to yield a pellet containing mitochondria from crushed nodules, it is unlikely that any of the succinate transport activity detected was due to contaminating mitochondria (8, 19). This is supported by the data in Table 5 which show that the mitochondrial succinate transport inhibitors 2-butylmalonate and phenylsuccinate (22, 23, 33) had little effect on the transport activity. Also, if the succinate transport activity observed was mitochondrial in origin, then fumarate should not have inhibited it (Table 5), as the dicarboxylate transporter in mitochondria is responsible for the transport of succinate and malate but not fumarate (33). The observation that bacteroids of strain GF31 have

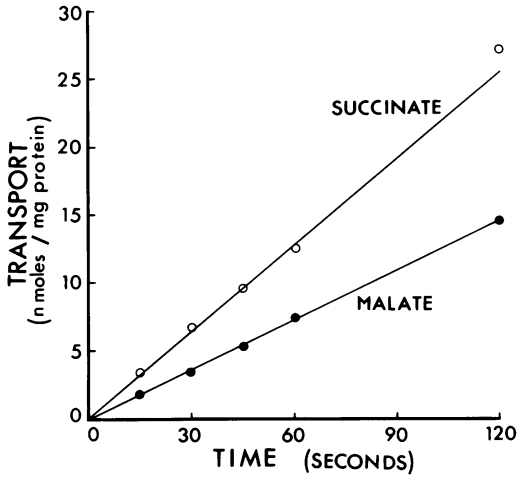

FIG. 2. Transport of succinate and malate by bacteroids of *R. leguminosarum* GF160. Bacteroids were prepared from pea root nodules 32 days after plant inoculation. The points shown are the mean values of triplicate assays. The succinate and malate concentrations in the assay mixtures were 50 and 20 μM, respectively.

no succinate transport activity also suggests that transport activity observed in bacteroid preparations of other strains was not due to succinate uptake by contaminating plant material which may have been present.

DISCUSSION

The data in this report strongly support the hypothesis that C_4-dicarboxylic acid transport activity in bacteroids is required for nitrogen fixation, but not for nodulation, by *R. leguminosarum*. Several lines of experimental evidence support this hypothesis.

Loss of the ability to transport C_4-dicarboxylic acids had no apparent effect on the ability of *R. leguminosarum* to infect and form nodules on the roots of pea plants (Table 3). Two mutant strains, GF31 and GFS5, lost the ability to fix N_2 symbiotically, whereas the third mutant, strain GF252, showed reduced symbiotic N_2-fixing activity (Tables 3 and 4). The loss of symbiotic N_2-fixing activity in strain GF31 is strongly associated with the Dct^- mutation, as the restoration of a Dct^+ phenotype in this strain resulted in the restoration of the ability to fix N_2 symbiotically (Table 3, strains GF310 and GF312). Also, the transfer of the Dct^- mutation from GF31 to GF160 resulted in the transfer of the inability to fix N_2 symbiotically (Table 3, strains GF131 and 132).

The ability of strain GF252 to fix N_2 symbiotically correlates with the ability of bacteroids of this strain to transport succinate and malate.

TABLE 4. Symbiotic properties of *R. leguminosarum* strains

Strain	Days after inoculation	Dry wt (g)[a]	Acetylene reduction[b]	Succinate transport[c]	Malate transport[c]
GF160	16	0.47 ± 0.13	37 ± 5	14.5 ± 0.7	ND[d]
	24	1.13 ± 0.13	26 ± 6	21.5 ± 0.8	10.2 ± 0.5
	31	2.97 ± 0.06	15 ± 3	13.1 ± 0.7	7.4 ± 0.6
GF252	16	0.42 ± 0.04	17 ± 5	6.2 ± 0.6	ND
	24	0.6 ± 0.10	19 ± 4	9.8 ± 1.6	5.6 ± 0.8
	31	1.8 ± 0.28	19 ± 2	8.2 ± 0.2	ND
GF31	32	0.46 ± 0.10	ND[e]	0	0
GFS18	32	0.53 ± 0.13	ND	6 ± 0.2	3.7 ± 0.1
Uninoculated	32	0.34 ± 0.11	0		

[a] Plants were grown in Leonard jars (three per jar) and inoculated as described in the text. Dry weight values (g) are expressed as the mean (for three plants) ± the standard deviation of five replicates, except for strain GF160 and GF252, 31 days after inoculation, when three and four replicates were used, respectively.

[b] Acetylene reduction activities are expressed as nanomoles of ethylene formed per hour per milligram of nodule wet weight ± the standard deviation of four replicates.

[c] Succinate and malate transport activities were determined at succinate and malate concentrations of 25 and 20 μM, respectively, as described in the text. Values are the means of three replicates ± the standard deviation of the mean and are expressed as nanomoles transported per minute per milligram of protein.

[d] ND, Not determined.

[e] In separate experiments, nodules from plants inoculated with GF31 had no acetylene-reducing activity.

However, the rate of succinate and malate transport in bacteroids of strain GF252 is lower than in bacteroids of the parent strain GF160, and likewise the symbiotic N_2-fixing activity of strain GF252 is lower than that of strain GF160 (Table 4). Also, bacteroids of the other Tn*5*-mediated Dct$^-$ mutant, strain GF31, were unable to transport succinate and malate, and this strain was unable to fix N_2 symbiotically (Table 4). The succinate transport activity of bacteroids from the Dct$^-$ mutant strain GFS5 has not yet been determined, but the Dct$^-$ mutation in this strain is strongly associated with the inability to fix N_2 symbiotically, as the Dct$^+$ transductant strain GFS51 forms effective nodules.

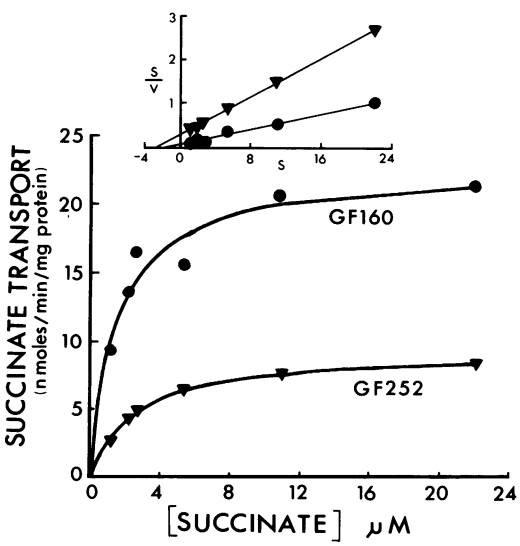

FIG. 3. Kinetics of succinate transport by bacteroids of *R. leguminosarum* GF160 and GF252. Bacteroids were prepared from pea root nodules grown for 23 days after plant inoculation. The points shown are the mean values of the data, and the lines drawn are those derived from regression analysis of these data. Bacteroids of *R. leguminosarum* GF160 (●) and GF252 (▼).

The succinate transport system in bacteroids of GF160 is similar to that found in the free-living bacteria (9). Both succinate transport activities exhibit saturation kinetics, and their apparent K_m values are between 1 and 3 μM. The two transport activities show the same specificity, and both are strongly inhibited by FCCP and DNP, whereas sodium arsenate has little effect on either (Table 5). These data therefore suggest that the same succinate transport system operates in free-living cells and in bacteroids. This is supported by the transport properties of the Dct$^-$ mutant strain GF31 as this strain was unable to transport succinate or malate both in free-living cells and in bacteroids (Tables 2 and 4).

We have previously shown that the succinate transport rate found in free-living cells is strongly dependent on the carbon source present in the medium in which they are grown (9). The succinate transport activity of GF160 bacteroids from nodules on plants sampled 23 days after inocula-

TABLE 5. Specificity and effect of metabolic inhibitor on succinate transport in bacteroids of *R. leguminosarum* GF160 and GF252[a]

Inhibitor	Concn	Relative succinate transport rate	
		GF160	GF252
None		1.00	1.00
Succinate	200 μM	0.03	0.03
2,2-Difluorosuccinate	200 μM	0.13	0.13
Malonate	200 μM	0.96	0.91
Fumarate	200 μM	0.03 (0.04)	0.04 (0.05)
Malate	200 μM	0.03	0.03
Citrate	200 μM	0.96	1.00
Oxaloacetate	200 μM	0.04	0.04
Phenylsuccinate	200 μM	0.80 (1.02)	0.70 (0.98)
N-Butylmalonate	200 μM	1.03 (1.17)	1.00 (1.14)
Arsenate	3 mM	0.95	ND[b]
DNP	200 μM	0.29	ND
FCCP	4 μM	0.02	ND

[a] Bacteroids were prepared from plants 31 days after inoculation as described in the text. The effects of arsenate, DNP, and FCCP were determined at a succinate concentration of 25 μM, and the uninhibited transport rate was 11.9 μmol/min per mg protein. All other assays were performed at a succinate concentration of 1.7 μM, and the uninhibited transport rates were 7 and 4 nmol/min per mg of protein for GF160 and GF252, respectively. Data from a separate experiment in which bacteroids were prepared from plants 24 days after inoculation are shown in parentheses.

[b] ND, Not determined.

tion was 22 nmol/min per mg of protein (Table 4). This rate is comparable with that observed in free-living bacteria induced for succinate transport (9). This observation is consistent with the view that the C_4-dicarboxylate transport system of bacteroids is induced to function in bacteroids.

The detection of succinate transport activity in strain GF252 bacteroids but not in free-living cells may reflect the unusual physiological conditions within the nodule. For example, the microaerophilic environment of the nodule and the essentially nondividing state of the bacteroids may allow expression of a dicarboxylic acid transport system which would not normally occur in free-living cells. This interpretation is supported by the data which show that the kinetics and specificity of the succinate transport activity of bacteroids of GF252 are very similar to the kinetics and specificity of the succinate transport activity determined for strain GF160, both in free-living cells and in bacteroids.

The leaky phenotype of the Dct^- mutant strains GF33, GF351, and GF252 (Tables 2 and 4) could be interpreted as indicating that a second succinate transport system is present. If this were so then the two transport systems must have at least one common component, as the Dct^- mutant strain GF31 is succinate and malate transport negative both in free-living cells and in the bacteroids. Also, if there are two transport systems, then the K_m and specificity of the bacteroid system must be very similar to that of operative free-living cells (Fig. 3 and Table 5). It would appear more likely that a single C_4-dicarboxylic acid transport system is present, and the phenotypes of the Dct^- mutants would indicate that the regulation of this system is complex. However, these possibilities cannot be fully resolved without further detailed genetic and biochemical analysis of the transport system.

The ineffective nodules formed by the Dct^- mutant strain GF31 differed from effective nodules in their smaller size and their apparent lack of leghemoglobin. Ultrastructurally, however, both nodule types were very similar, indicating that the transport system is only necessary during the final stages of nodule development. This is consistent with the involvement of C_4-dicarboxylate transport in the N_2 fixation process, although it does not identify the reaction process involved. Three roles for C_4-dicarboxylate acids clearly relevant to nitrogen fixation are the direct provision of energy for the N_2 reduction process, the production of porphyrin for leghemoglobin synthesis, and an anaplerotic role in supporting the tricarboxylic acid cycle.

Succinate, fumarate, and malate stimulate N_2 fixation and respiration by isolated pea bacteroids (F. Houwaad, Ph.D. thesis, Agricultural University, Waseningen, The Netherlands, 1979). In light of those data, our results strongly suggest that these compounds also serve as energy sources for N_2 fixation by bacteroids in pea nodules.

Alternatively, as bacteroids seem to synthesize the heme moiety of leghemoglobin (1, 6, 12, 20), and as succinyl coenzyme A is an essential precursor in the synthesis of heme, it is probable

that the bacteroids utilize dicarboxylic acids both for heme synthesis and as carbon sources. The inability of bacteroids to transport C_4-dicarboxylic acids may therefore result in an inability to synthesize heme, thus resulting in ineffective nodules.

The C_4-dicarboxylic acids may also be required in an anaplerotic role, as the tricarboxylic acid cycle is an amphibolic pathway and there is evidence that the glyoxylate cycle does not operate in bacteroids of *R. leguminosarum* (14). It is unlikely that ineffectiveness is a result of an overall permeability defect because a specific transport-defective phenotype is associated with a Dct^- mutation (Table 2).

Although the results of the present study strongly suggest that C_4-dicarboxylic acid(s) is utilized by bacteroids in N_2-fixing pea root nodules, it is perhaps surprising that alternate carbon sources cannot replace C_4-dicarboxylic acid(s). This may reflect stringent control of carbohydrate metabolism in the host cell or fastidious requirements of the bacteroid. Also, the peribacteroid membrane may limit the accessibility of carbon sources to the bacteroid. However, many amino acid auxotrophs form effective nodules, and therefore, at least some amino acids produced in the plant can cross the peribacteroid membrane (30).

Other workers have obtained data which are consistent with ours. A C_4-dicarboxylic acid transport system is present both in free-living and bacteroid forms of two other strains of *R. leguminosarum* (11; G. de Vries, Ph.D. thesis, Botanical Laboratory, Leiden, Holland, 1981). Also, Glenn and Brewin (10) have shown that mutants of *R. leguminosarum* with reduced succinate uptake activity formed ineffective nodules. In a more detailed study, Ronson et al. (27) have presented evidence which indicates that succinate, fumarate, and malate are also transported by the same system in *Rhizobium trifolii*. In addition, they showed that C_4-dicarboxylic acid transport mutants were ineffective and also that the ineffective nodules were structurally very similar to wild-type effective nodules.

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