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Aspartate Aminotransferase Activity Is Required for Aspartate Catabolism and Symbiotic Nitrogen Fixation in *Rhizobium meliloti*†

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A mutant of *Rhizobium meliloti*, 4R3, which is unable to grow on aspartate has been isolated. The defect is specific to aspartate utilization, since 4R3 is not an auxotroph and grows as well as its parent strain on other carbon and nitrogen sources. The defect was correlated with an inability to fix nitrogen within nodules formed on alfalfa. Transport of aspartate into the mutant cells was found to be normal. Analysis of enzymes involved in aspartate catabolism showed a significantly lower level of aspartate aminotransferase activity in cell extracts of 4R3 than in the wild type. Two unrelated regions identified from a genomic cosmid bank each complemented the aspartate catabolism and symbiotic defects in 4R3. One of the cosmids was found to encode an aspartate aminotransferase enzyme and resulted in restoration of aspartate aminotransferase activity in the mutant. Analysis of the region cloned in this cosmid by transposon mutagenesis showed that mutations within this region generate the original mutant phenotypes. The second type of cosmid was found to encode an aromatic aminotransferase enzyme and resulted in highly elevated levels of aromatic aminotransferase activity. This enzyme apparently compensated for the mutation by its ability to partially utilize aspartate as a substrate. These findings demonstrate that *R. meliloti* contains an aspartate aminotransferase activity required for symbiotic nitrogen fixation and implicate aspartate as an essential substrate for bacteria in the nodule.

Rhizobium meliloti, a gram-negative soil bacterium, fixes atmospheric nitrogen within nodules developed on the roots of alfalfa (*Medicago sativa*). Establishment of the symbiosis involves a series of steps beginning with root hair curling and penetration of bacteria within the curl. The bacteria move through a specialized tubular infection thread to the root cortex, where they are released into the cortical cells. There, they undergo a limited number of cell divisions before differentiating into large, elongated bacteriods enclosed within a host-derived peribacteroidal membrane. The host plant supplies the bacteria with metabolic substrates and, in return, receives reduced nitrogen in the form of ammonia.

Identification of carbon and nitrogen substrates supplied by the plant to sustain bacterial growth and nitrogen fixation within the nodule is fundamental to elucidation of the metabolic pathways used by both partners during the symbiosis. Little is known of the carbon and nitrogen sources used by the bacteria during growth within the infection thread or during the initial stages of their proliferation within the host cortical cells. One or more of the C_4-dicarboxylate tricarboxylic acid (TCA) cycle intermediates, succinate, fumarate, and malate, have generally been considered to be the principal carbon and energy substrates provided by the plant to the mature bacteroids (7, 40).

The major evidence implicating the TCA cycle intermediates has come from genetic studies in which mutants of *Rhizobium* spp. lacking defined metabolic capabilities have been tested for symbiotic nitrogen fixation ability. These studies have generally demonstrated that enzymes involved in carbohydrate catabolism are dispensable (18, 35), whereas those required for functions of the TCA cycle are necessary for effective bacteroid metabolism (10, 16). Evidence uniquely implicating C_4-dicarboxylates has come from studies of dicarboxylate transport (*dct*) mutants (11, 23, 33, 41, 42, 46). These mutants grow well on a variety of carbon substrates, but they are unable to utilize C_4-dicarboxylates because of their inability to transport them into the cell. Mutations in the *dctA* gene, which encodes the structural protein for dicarboxylate transport found in the membrane, cause the formation of ineffective nodules, demonstrating that dicarboxylates are an essential substrate for the bacteria during the symbiosis. Strains mutated in *dctB* and *dctD*, which are regulatory genes controlling the induction of *dctA*, have a partial ability to fix nitrogen, despite their inability to transport dicarboxylates in the free-living state. The reason for this discrepancy is not clear, though it is possible that genes other than *dctB* and *dctD* can serve to induce *dctA* in the symbiotic state. Strains mutated in the *ntrA* gene are also incapable of dicarboxylate transport (34, 42, 46). This phenotype is caused by the inability of *ntrA* mutants to initiate transcription of the *dctA* gene.

Previously, we have shown that aspartate, like succinate, fumarate, and malate, requires the dicarboxylate transport system for transport into *R. meliloti* for use as a carbon source. For this reason, *dct* and *ntrA* mutants cannot utilize aspartate (42, 43). Those observations suggested that aspartate could play a role in the symbiosis, possibly instead of the TCA cycle intermediates. In this report we describe an *R. meliloti* mutant which is specifically blocked in aspartate catabolism as a result of a mutation affecting aspartate aminotransferase (AspAT) activity. The same mutation causes the mutant to form symbiotically defective nodules. These results suggest that aspartate may serve as an essential substrate for bacteria within the nodule.

MATERIALS AND METHODS

Strains, plasmids, and media. Bacterial strains and plasmids are described in Table 1. *R. meliloti* and *Agrobacterium tumefaciens* strains were grown on TYC medium (3) or M9 minimal medium (28). NH_4Cl was omitted from M9 medium for growth tests using other nitrogen sources. Antibiotics

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Characteristics	Source or reference
R. meliloti		
JJ1c10	Wild-type parent, derivative of IZ450, Fix$^+$ Rif^r	38
4R3	Mutant derived from JJ1c10, unable to utilize aspartate, lacks aspartate aminotransferase activity, contains Tn*5*, Fix$^-$ Km^r Rif^r	This work
4F6	*dctA* mutant derived from JJ1c10 by Tn*5* mutagenesis, Dct$^-$ Fix$^-$ Km^r Rif^r	43
R678, R679	JJ1c10::Tn*5*(Gm)-b148, JJ1c10::Tn*5*(Gm)-b69, JJ1c10 derivatives containing genomic Tn*5*(Gm) inserts in region I introduced by recombination, unable to utilize aspartate, contains pBB97, Fix$^-$ Gm^r Km^r Rif^r	This work
R680	JJ1c10::Tn*5*-c2, JJ1c10 derivative containing genomic Tn*5* insert in region II introduced by recombination, contains pPH1JI, Fix$^+$ Km^r Gm^r Rif^r	This work
E. coli		
HB101	*E. coli* host strain, *supE44 hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	H. W. Boyer (4)
DH5α	*E. coli* host strain, *endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* Δ(*argF-lacZYA*)U169 φ80d*lacZ* M15 λ$^-$	J. Jessee, Bethesda Research Laboratories, Inc.
A. tumefaciens		
UBAPF-1	Plasmid-free derivative of *A. tumefaciens* C58, Sm^r Rif^r	M. Hynes (21)
Plasmids		
pLAFR1	Cosmid cloning vehicle, IncP1, Tc^r	F. M. Ausubel (15)
pVK100	Cosmid cloning vehicle, IncP1; Tc^r Km^r	E. W. Nester (26)
pLAFR1 clone bank	JJ1c10 *Eco*RI-digested DNA in cosmid vector pLAFR1, Tc^r	L. Barran (38)
pVK100 clone bank	JJ1c10 *Hin*dIII-digested DNA in cosmid vector pVK100, Tc^r	L. Barran (38)
pRK2013	ColE1 replicon with RK2 transfer genes, Km^r	D. R. Helinski (12)
pRK600	pRK2013::Tn*9* derivative, Cm^r	T. M. Finan (14)
pPH1JI	IncP1, Gm^r	G. Ditta (36)
pBB97	RP4 derivative, IncP1, Ap^r Km^r	43
pRK310	Broad-host-range cloning vehicle, Tc^r	G. Ditta (8)
pUC19	*E. coli* cloning vehicle, Ap^r	45
pCHR81	Tn*5* delivery vehicle, temperature-sensitive replication, R388 derivative, Tp^r Km^r	C. Sasakawa (37)
pCHR84	Tn*5*(Gm) delivery vehicle, temperature-sensitive replication, R388 derivative, Tp^r Gm^r	C. Sasakawa (37)
pBB131, pBB133, pBB134	pVK100 cosmids complementing the aspartate utilization defect in 4R3, region I, Tc^r	This work
pBB135	pLAFR1 cosmid complementing the aspartate utilization defect in 4R3, region I, Tc^r	This work
pVR8	pRK310 carrying a 7.3-kb *Hin*dIII fragment from region I complementing the 4R3 aspartate utilization defect, Tc^r	This work
pBB132	pVK100 cosmid complementing the aspartate utilization defect in 4R3, region II, Tc^r	This work
pBB130	pLAFR1 cosmid complementing the aspartate utilization defect in 4R3, region II, Tc^r	This work
pVR15	pRK310 carrying a 11.0-kb *Hin*dIII fragment from region II complementing the 4R3 aspartate utilization defect, Tc^r	This work

used were rifampin (100 μg/ml), tetracycline (5 μg/ml), kanamycin (40 μg/ml), and gentamicin (25 μg/ml). *Escherichia coli* strains were grown on LB medium (28). Antibiotics used for *E. coli* were streptomycin (100 μg/ml), tetracycline (10 μg/ml), kanamycin (20 μg/ml), ampicillin (20 μg/ml), chloramphenicol (10 μg/ml), and trimethoprim (10 μg/ml). Measurements of growth rates of *R. meliloti* in liquid media were done at 30°C in M9 containing 20 mM carbon source and 50 μg of yeast extract (Difco, Detroit, Mich.) per ml. Aspartate as a nitrogen source was used at 4 mM. A_{620} was used to monitor growth. Growth comparisons on solid media were done by streaking strains and controls on M9 agar plates and observing the relative sizes of single colonies each day. Transduction of Tn*5* from 4R3 to *R. meliloti* JJ1c10 was done as described by Finan et al. (13).

Genetic manipulations. Triparental matings to introduce pVK100, pLAFR1, or pRK310 derivatives from *E. coli* into *R. meliloti* or *A. tumefaciens* were done by using pRK2013 or pRK600 as described previously (9). pBB97 in *R. meliloti* JJ1c10 was used as a helper plasmid in triparental matings when cosmids were mobilized from *R. meliloti* into *E. coli*. Restriction enzyme digestions, agarose gel electrophoresis, purification of individual restriction fragments, and their cloning were done by standard methods compiled by Maniatis et al. (28). Hybridizations to localize region I and region II were done as described previously for pBB107 (43). Localized Tn*5*(Gm) and Tn*5* mutagenesis of cosmids was done by using temperature-sensitive plasmids pCHR84 and pCHR81, respectively (37). A culture of HB101 carrying the cosmid and either pCHR84 or pCHR81 was grown at 42°C in LB containing tetracycline to cure the temperature-sensitive plasmid and then diluted into fresh medium containing gentamicin or kanamycin to select cells in which transposition had occurred. The culture was used to prepare plasmid, which was then used to transform *E. coli* DH5α. Selection for the appropriate transposon gave predominantly cosmids containing transposon insertions. Transposons were recombined into the *R. meliloti* genome by the marker exchange technique described by Ruvkun and Ausubel (36) except that pBB97 was used instead of pPH1JI as a P-group plasmid to

displace cosmids by incompatibility when recombining Tn*5*(Gm) into the genome.

Plant tests. Plants were grown on slopes of nitrogen-free plant nutrient agar and inoculated with the *R. meliloti* cells to be tested as described previously (38). Nitrogen fixation was assayed 28 days after inoculation by acetylene reduction.

Transport assays. Transport assays were performed as described previously (43), using 50 μM [2,3-^{14}C]succinate (1.55 GBq/mmol) or 4 mM L-[U-^{14}C]aspartate (46 MBq/mmol) (Amersham Canada). Bacteria were grown in M9 medium with 20 mM glucose and induced for 1 h in 20 mM succinate or aspartate prior to assays of succinate or aspartate transport.

Enzyme assays. Crude protein extracts of late-log-phase cells of *R. meliloti* were prepared by sonication in 25 mM Tris-PO_4 (pH 6.9)–20 μM EDTA–20 μM pyridoxal monophosphate–50 μM dithiothreitol. Malate dehydrogenase was assayed as described by Bergmeyer and Bernt (2). NADH oxidation was monitored at 340 nm, and the activity was determined by using a molar extinction coefficient of 6220. AspAT was assayed by the UV method described by Bergmeyer and Bernt (1). The fumarase assay used was as described by Hill and Bradshaw (20). Increase in A_{240} was recorded, and the amount of fumarate produced was calculated by using a molar extinction coefficient of 2530. Aspartase activity was measured by the spectrophotometric method of Williams and Lartigue (44). Aspartase activity in the extracts was also tested for by assaying for ammonia production as described by Kaplan (25). Aromatic (tyrosine) aminotransferase (AroAT) activity was spectrophotometrically assayed by a modified Diamondstone method (19). The molar extinction coefficient used to calculate the amount of *p*-hydroxybenzaldehyde produced was 19900.

Electrophoretic separation of aminotransferase activities. AspAT and AroAT enzyme activities were detected after separation of proteins in crude extracts, using a nondenaturing polyacrylamide gel system as described by Davis (5). Approximately 100 μg of crude protein extract was loaded in each sample slot. AspAT activity was detected by staining the gel in the dark at room temperature as described by Rej et al. (32). The staining mixture contained 5 mM aspartate, 2.5 mM α-ketoglutarate, 4 mM NAD, 1 mM ADP, 60 U of glutamic dehydrogenase per ml, 0.49 U of diaphorase per ml, and 1.49 mM iodonitrotetrazolium chloride. Tyrosine, phenylalanine, or tryptophan was used in place of aspartate to detect aminotransferase activity on aromatic amino acid substrates. Leucine or isoleucine was used to detect aminotransferase activity on branched-chain amino acid substrates. Activity bands appeared as dark pink areas against a clear background.

RESULTS

Derivation of an *R. meliloti* mutant unable to utilize aspartate. We have previously described the derivation of a collection of symbiotically defective mutants of *R. meliloti* JJ1c10 by Tn*5* mutagenesis (38). These mutants were screened for their ability to grow on each of the C_4-dicarboxylates, aspartate, succinate, fumarate, and malate. Two classes were obtained, one unable to grow on any of the dicarboxylates and one unable to grow on aspartate but able to grow on succinate, fumarate, and malate. The first class consisted of *dct* and *ntrA* mutants which have been described previously (42, 43). The second class was represented by only one mutant, 4R3, which we describe here.

Growth rate comparisons in minimal media demonstrated that 4R3 grows at the same rate as the parent strain with mannitol, glucose, L-arabinose, succinate, fumarate, malate, glutamate, lysine, leucine, isoleucine, γ-aminobutyrate, or α-ketoglutarate as a carbon source and ammonia as a nitrogen source. The mutant shows no appreciable growth with aspartate as a carbon source (doubling time of >60 h, compared with 5.5 h for the wild type). Similarly, in nitrogen source comparisons the mutant and wild type grew at the same rate as the parent with mannitol as a carbon source and glutamate or glutamine as a nitrogen source, but growth with aspartate as a nitrogen source resulted in significantly reduced growth of 4R3 (doubling time of 11 h, compared with 5 h for the wild type).

TABLE 2. Symbiotic properties of mutant 4R3 and derivatives[a]

Inoculum	No. of nodules/plant	Top dry wt (mg/plant)	Acetylene reduction (nmols/h/plant)
JJ1c10	5.6	11.6	98.1
None	0	3.4	1.9
4R3	20.0	4.3	6.2
4R3(pBB131)	3.8	8.0	82.7
4R3(pBB132)	2.4	7.3	67.1
4R3(pVR8)	4.0	9.7	117.8
4R3(pVR15)	4.5	7.8	92.5

[a] Data are averages from eight or more plants tested 28 days after inoculation.

The symbiotic properties of 4R3 are summarized in Table 2. Plants inoculated with this mutant were stunted and turned yellow within 3 to 5 weeks. Compared with the wild type, the ineffective nodules were smaller in size and pale-white in color and tended to be produced in higher numbers. The rate of acetylene reduction by 4R3-inoculated plants was very low, indicating that the mutant is Fix$^-$.

Molecular analysis of the regions complementing 4R3. Although 4R3 was identified from a collection of Fix$^-$ mutants obtained after Tn*5* mutagenesis, it was found that ΦM12 transduction of Tn*5* from this mutant to the parent strain did not result in transfer of the aspartate utilization or Fix$^-$ phenotype. Thus, the mutation(s) of interest was not due to Tn*5*. To identify the genomic DNA responsible for the aspartate utilization defect in 4R3, pLAFR1 and pVK100 cosmid banks of *R. meliloti* JJ1c10 DNA in *E. coli* were mated with 4R3, and transconjugants capable of utilizing aspartate for growth were picked. Their complementing cosmids were mobilized back into *E. coli* HB101 for molecular characterization.

Restriction mapping of the DNA cloned in these cosmids established that their DNA originated from two different genomic regions, referred to here as region I and region II, represented by cosmids pBB131 and pBB132, respectively (Fig. 1). Cosmids from both regions complemented the aspartate utilization defect when mobilized back into *R. meliloti* 4R3, although the growth rate on aspartate was not completely restored to wild-type levels by region II. Hybridization analysis showed that the DNAs cloned in pBB131 and pBB132 are nonhomologous and are located in the *R. meliloti* chromosome (results not shown). Plants inoculated with 4R3 carrying cosmids from either region were green and fixed N_2 at the same rate as those inoculated with the wild-type strain (Table 2).

Usually, the phenotypes imparted by a mutated gene can be complemented by introduction of the corresponding wild-type gene into the cell. Our ability to complement the 4R3 mutant phenotypes with two different DNA regions

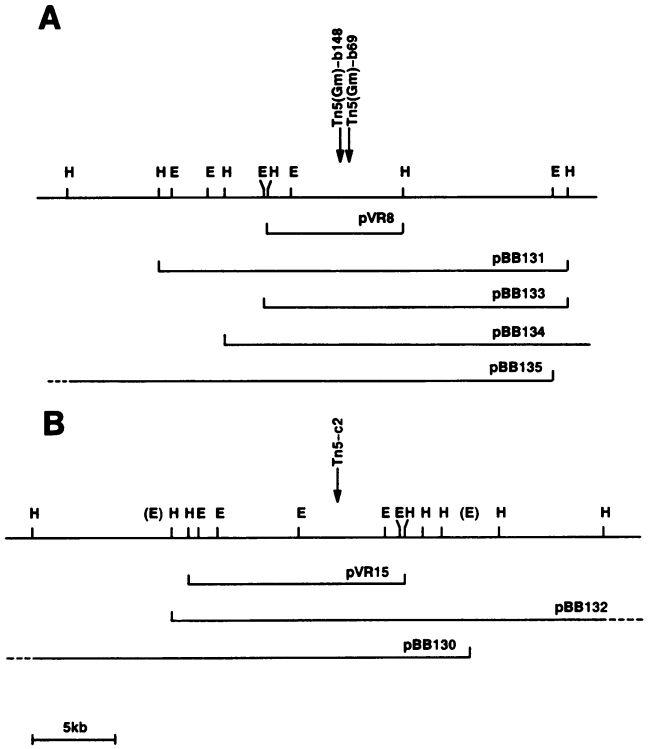

FIG. 1. Regions of the *R. meliloti* genome which complement the aspartate utilization and symbiotic defects of mutant 4R3. (A) Region I; (B) region II. The restriction map of each region is shown. E, *Eco*RI site; H, *Hin*dIII site. In panel B, the *Eco*RI sites are mapped only within the 11.0-kb *Hin*dIII fragment cloned in pVR15; outside this fragment there are at least 10 other *Eco*RI sites, as indicated by (E). Cosmids used to define these regions and plasmids with subcloned fragments which complement the mutant phenotypes of 4R3 are shown at the bottom. The arrows indicate the positions of Tn*5*(Gm) and Tn*5* insertions resulting in loss of ability of these regions to complement the 4R3 phenotypes.

suggested that one of these regions contained the expected wild-type allele, while the other complemented by another mechanism. In an attempt to distinguish between the two regions, they were analyzed by subcloning and by localized Tn*5* mutagenesis.

Region I cosmids contained several *Hin*dIII fragments in common. By subcloning these into pRK310 and testing their ability to complement the mutant phenotypes of 4R3, the complementing region was localized to a 7.3-kb fragment (pVR8; Fig. 1A). Also, localized mutagenesis was used to derive Tn*5*(Gm)-carrying derivatives of cosmid pBB131 which had lost the ability to complement the aspartate utilization defect of 4R3. Two such cosmid derivatives analyzed were found to have Tn*5*(Gm) inserted into the 7.3-kb *Hin*dIII fragment. These Tn*5*(Gm) inserts were introduced into the chromosome of the wild-type strain, JJ1c10, by marker exchange. The resultant derivatives, R678 and R679, were unable to utilize aspartate and were symbiotically defective. One of these derivatives, R679, was used as a recipient for pBB131 and pBB132, to test for complementation of its phenotypes by region I and region II. Both cosmids restored the ability of the strain to grow on aspartate and fix nitrogen on plants. These experiments demonstrated that a defined mutation within region I can simultaneously produce both phenotypes and is probably the site of the relevant mutation in 4R3.

A similar analysis of region II was done. Subcloning restriction fragments into pRK310 demonstrated that the region complementing the 4R3 phenotypes was in an 11-kb *Hin*dIII fragment within the overlapping portion of both cosmids containing the region (pVR15; Fig. 1B). Localized mutagenesis was done as described for region I, using Tn*5* to obtain a pBB132::Tn*5* cosmid derivative which was unable to complement the 4R3 phenotypes. This Tn*5* insert was mapped within the 11-kb *Hin*dIII fragment. However, when introduced into the chromosome of the wild-type strain by marker exchange, this insertion did not affect the ability of the bacteria to grow on aspartate. The derivative, R680, was also fully effective in the symbiosis. These results indicate that the gene responsible for complementation by region II is not itself required for aspartate utilization or symbiotic nitrogen fixation. It must complement the 4R3 mutant phenotypes by an indirect mechanism related to the fact that the cloned region is present on a cosmid.

Mutant 4R3 can transport aspartate. To systematically search for the physiological lesion caused by the mutation in 4R3, we first tested the ability of the mutant to transport aspartate into the cell. Since transport of succinate and aspartate is mediated by the dicarboxylate transport (*dct*) system, uptake of these compounds was compared by using 4R3, *dctA* mutant 4F6, and the wild-type strain. Succinate uptake was unaffected in 4R3 compared with the wild-type *R. meliloti* JJ1c10 (Fig. 2A). The initial rate of aspartate uptake by 4R3 matched that of the wild type, whereas 4F6 exhibited a decreased rate (Fig. 2B). The absence of initial rate differences established that 4R3 is not defective in aspartate transport. With longer times, the rate of aspartate uptake gradually decreased (Fig. 2B). Comparison of 4R3 and JJ1c10 incorporation for 180 min showed that the rate of aspartate uptake by 4R3 continued to decrease until it plateaued (data not shown). The 4R3 cells did not grow during this time. These data are consistent with a metabolic block existing in 4R3, such that aspartate which is taken up into the cells is not catabolized.

Mutant 4R3 exhibits a decreased level of AspAT activity. On the basis of the growth characteristics and transport data, it was apparent that mutant 4R3 is defective in one of the enzymes involved with aspartate catabolism. In bacteria, aspartase (aspartate ammonia lyase) and AspAT are the two key enzymes known to mediate aspartate catabolism to TCA cycle intermediates, as shown in Fig. 3. The levels of these enzymes were measured in crude extracts of *R. meliloti* wild type, mutant 4R3, and the derivatives of 4R3 carrying region I, 4R3 (pBB131), and region II, 4R3 (pBB132) (Table 3). Two TCA cycle enzymes, malate dehydrogenase and fumarase, were also assayed.

Aspartase activity was not detectable in any of the *R. meliloti* extracts, including the wild type. We tested cells grown in TYC and in minimal medium with aspartate, fumarate, or mannitol as a carbon source. Extracts were tested both by a spectrophotometric assay for fumarate production and by quantitation of ammonia production. In contrast, control extracts of *E. coli* HB101 showed significant aspartase activity (about 400 nmol/min/mg of protein).

A significant difference in AspAT activity was found between mutant 4R3 and wild-type extracts; the level of 4R3 AspAT activity was reduced to about 40% of the wild-type level. Both pBB131 and pBB132 restored the AspAT activity in 4R3 to levels exceeding that of the wild type. Since it is known that aminotransferases often exhibit overlapping sub-

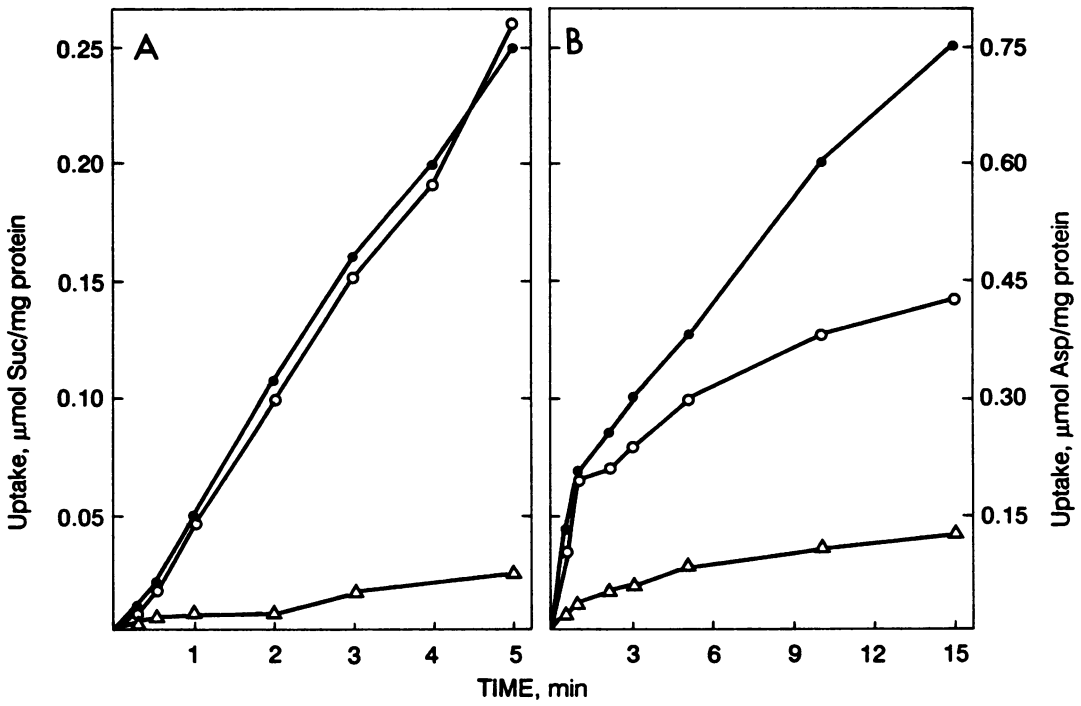

FIG. 2. Uptake of succinate (A) and aspartate (B) by JJ1c10 (●), 4R3 (○), and *dctA* mutant 4F6 (△). Strains were grown in M9 medium with glucose as the carbon source and ammonia as the nitrogen source. They were incubated for 1 h in 20 mM succinate to induce succinate uptake or in 20 mM aspartate to induce aspartate uptake. Uptake was assayed by using 50 μM succinate or 4 mM aspartate and ^{14}C-labeled substrates.

strate specificities, we also assayed AroAT activity, using tyrosine, to test its possible contribution to the AspAT assay. AroAT activity was much higher in 4R3 containing the region II cosmid and also significantly higher in 4R3 containing region I. Mutant 4R3 and the wild type did not differ significantly in AroAT activity.

Our interpretation of these results is that the mutation in 4R3 has resulted in the absence of active AspAT enzyme. The region I cosmid, pBB131, has restored the AspAT levels, and in fact has caused an overproduction of AspAT. The increased AspAT enzyme levels in 4R3 (pBB131) were also detectable as an increased AroAT activity in the extracts, since the AspAT enzyme is partially active with tyrosine as a substrate. Region II complements the 4R3

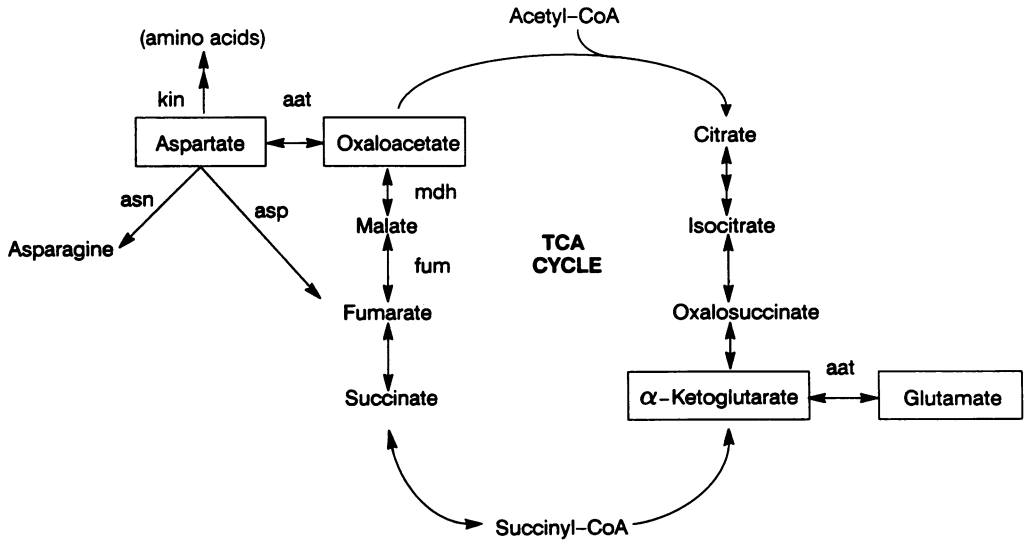

FIG. 3. Aspartate metabolic pathways and their relationship to the TCA cycle. Substrates for aspartate aminotransferase are boxed. aat, aspartate aminotransferase; asp, aspartase; asn, asparagine synthetase; fum, fumarase; kin, aspartate kinase; mdh, malate dehydrogenase; CoA, coenzyme A.

TABLE 3. In vitro enzyme activities in extracts of *R. meliloti* and *A. tumefaciens* strains

Strain	Description	Medium[a]	Enzyme activity (nmol/min/mg of protein)[b]				
			Malate dehydrogenase	Fumarase	Aspartase	AspAT	AroAT[c]
JJ1c10	Wild type	TYC	3,014	447	<20	152	120
4R3	Mutant	TYC	3,129	469	<20	65	150
4R3(pBB131)	Region I, complemented	TYC	3,303	457	<20	294	250
4R3(pBB132)	Region II, complemented	TYC	3,392	501	<20	178	399
JJ1c10	Wild type	M9	2,910	267	<20	177	ND
4R3	Mutant	M9	3,400	288	<20	80	ND
UBAPF-1	Wild-type *A. tumefaciens*	M9	2,338	ND	ND	153	101
UBAPF-1(pVR8)	Region I in *A. tumefaciens*	M9	2,719	ND	ND	294	115
UBAPF-1(pVR15)	Region II in *A. tumefaciens*	M9	2,375	ND	ND	189	193

[a] Medium in which the cells were grown for preparation of enzyme extracts. M9 medium was supplemented with 20 mM mannitol as a carbon source.
[b] Mean of at least two experiments. ND, Not determined.
[c] Assayed by using tyrosine as a substrate.

defects by an analogous mechanism; when present on a cosmid, it results in a very high level of AroAT enzyme activity. The wide substrate specificity of the AroAT enzyme is able to compensate for the AspAT enzyme lacking in 4R3, since AroAT has partial activity with aspartate as a substrate.

To better distinguish between the aminotransferases, they were localized by enzyme-specific staining after separation of protein extracts by polyacrylamide gel electrophoresis (Fig. 4). This technique was found to produce several bands for each aminotransferase tested, some of which were variable or dependent upon the growth media. Cells grown in minimal media gave extracts which showed one major AroAT band when stained with aromatic amino acids such as tyrosine. When aspartate was used as a substrate, a band corresponding to the AroAT band was produced, plus an aspartate-specific AspAT band below it. Staining of this AspAT band was markedly less intense than that of the AroAT band. This difference is not quantitatively consistent with the relative activities of AspAT and AroAT with aspartate as a substrate which were found in the in vitro assays of cell extracts described above. The light staining of the AspAT band may be due to differences in the reaction conditions in the gel compared with those used in the in vitro assays, or possibly the AspAT is less stable than the AroAT during electrophoresis. Staining for branched-chain aminotransferases, with leucine or isoleucine as a substrate, showed bands different from those produced by the AspAT and AroAT enzymes, with no differences among the four extracts (results not shown).

The AspAT band differed in intensity among the four extracts. The 4R3 extract showed no staining at the position corresponding to the AspAT band in the wild-type extract. The extract from 4R3 containing region I DNA, 4R3 (pBB131), showed the most pronounced band. These results agree with the AspAT results obtained for in vitro enzymatic assays as described above (Table 3) and confirm that 4R3 is lacking an AspAT activity which is restored by region I. Staining with the aromatic amino acid tyrosine, tryptophan, or phenylalanine as a substrate produced a very strong AroAT band in the lane containing the extract of 4R3 (pBB132) cells compared with the extracts of *R. meliloti* JJ1c10, mutant 4R3, and 4R3 (pBB131). This result confirms that region II complements because of excess activity of an

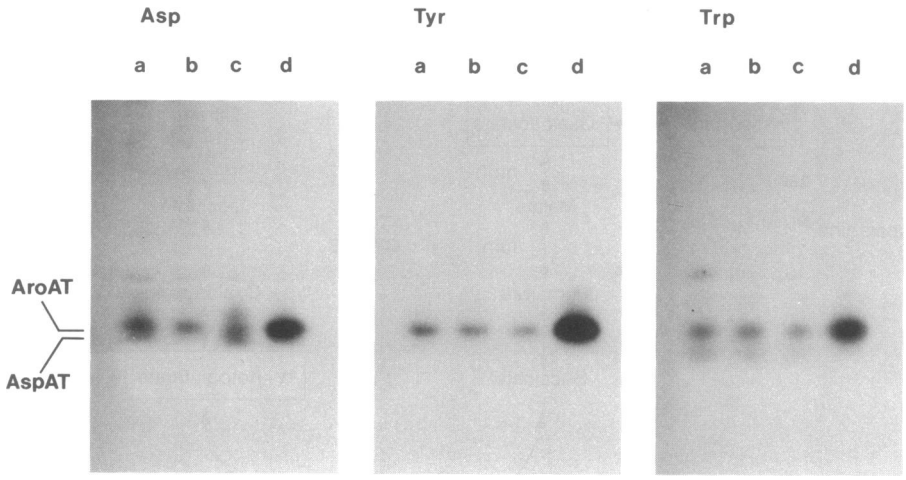

FIG. 4. Electrophoretic separation of AspAT and AroAT activities. Protein extracts were prepared by sonication, electrophoresed through 7.5% polyacrylamide gels at 4°C, and stained by enzyme-specific reactions as described in Materials and Methods. Extracts were made from cells grown in M9 medium with 20 mM mannitol as a carbon source. Lanes: a, *R. meliloti* JJ1c10; b, mutant 4R3; c, 4R3 complemented by region I [4R3(pBB131)]; d, 4R3 complemented by region II [4R3(pBB132)]. The amino acids used as substrates for the aminotransferase reactions are shown above the panels. The positions of bands identified as AroAT and AspAT are indicated.

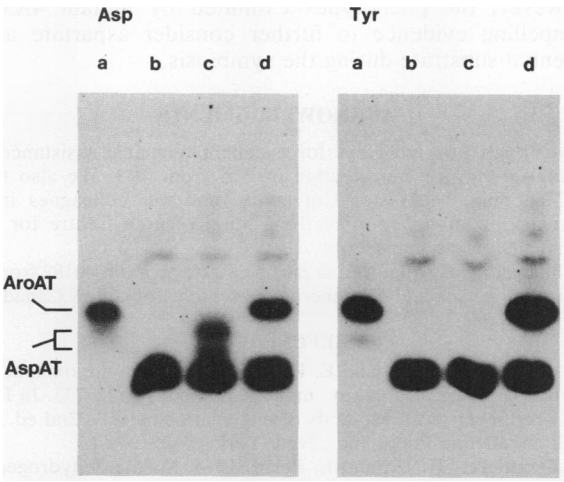

FIG. 5. Electrophoretic separation of AspAT and AroAT activities associated with *R. meliloti* region I and region II transferred to *A. tumefaciens*. Extracts were prepared and separated in polyacrylamide gels as described for Fig. 4. Lanes: a, *R. meliloti* JJ1c10; b, *A. tumefaciens* UBAPF-1 (wild type); c, *A. tumefaciens* carrying region I [UBAPF-1(pVR8)]; d, *A. tumefaciens* carrying region II [UBAPF-1(pVR15)]. The amino acids used as substrates for the aminotransferase reactions are shown above the panels. The positions of bands identified as AroAT and AspAT are indicated.

AroAT enzyme which is electrophoretically separable and enzymatically distinct from that affected by the original mutation.

To determine whether region I and region II encode structural genes associated with the aminotransferase activities, plasmids pVR8 and pVR15 containing the 7.3- and 11.0-kb *Hin*dIII fragments from these regions were transferred to *A. tumefaciens*. Assays of the aminotransferase activities of extracts from the transconjugants showed that pVR8 imparted enhanced AspAT activity and pVR15 imparted enhanced AroAT activity (Table 3). These extracts were also compared by activity staining after separation by polyacrylamide gel electrophoresis (Fig. 5). The gels showed a strongly staining, high-mobility band in all lanes containing extracts from *A. tumefaciens*, including the parent strain. This band represented most of the *A. tumefaciens* aminotransferase activity obtained with either aspartate or tyrosine as a substrate. In the extract from the strain carrying the 11.0-kb *Hin*dIII fragment from region II, there was a band corresponding to the AroAT band from the *R. meliloti* extracts (Fig. 5, lane d). This band stained with either aspartate or tyrosine, although tyrosine was found to be the better substrate.

The *A. tumefaciens* strain carrying the cloned region I showed AspAT activities in two bands which stained with aspartate and were not present in the parent (Fig. 5, lane c). The upper band corresponded in position to the faint AspAT band present in *R. meliloti* extracts. The lower band did not correspond to a band from the *R. meliloti* extract. However, the interpretation of this lower band difference is unclear; this portion of the *R. meliloti* lane has been observed to contain an enzyme activity which inhibits the aminotransferase staining reaction. We have also compared extracts of *A. tumefaciens* carrying cosmids pBB131 (region I) and pBB132 (region II) and the derivatives of these cosmids in which Tn*5* insertions have resulted in loss of ability to complement the aspartate utilization and nitrogen fixation defects. For both regions, the transposon insertions result in absence of the *R. meliloti* aminotransferase bands described above from the *A. tumefaciens* recipients, including both the upper and lower AspAT bands associated with pVR8 and pBB131 (data not shown).

DISCUSSION

In this study, we have described an *R. meliloti* mutant which was initially isolated as symbiotically defective and subsequently found to be specifically incapable of growth on aspartate. Both phenotypes have been attributed to mutation of a region of DNA necessary for AspAT activity. Interestingly, the mutation can be complemented either by the introduction of the wild-type region, referred to here as region I, or by a second unlinked and nonhomologous region, region II, which imparts a high level of AroAT activity. The AroAT enzyme must compensate for the absence of AspAT enzyme by its ability to utilize aspartate as a secondary substrate. By transferring region I and region II to *A. tumefaciens*, it was found that these regions contain structural genes for AspAT and AroAT enzymes, respectively. Two bands of AspAT activity were observed in the *A. tumefaciens* transconjugants containing region I. It seems unlikely that two genes encoding AspAT enzymes are present in this region. Among other possible explanations for this result is the presence of two translational start sites in the AspAT gene or the possibility that the enzyme encoded by this gene is subject to posttranslational modification.

The results described here demonstrate that AspAT activity is required by *R. meliloti* for growth on aspartate. Many bacteria which grow on aspartate, including *E. coli*, synthesize aspartase, which converts aspartate to fumarate. No aspartase activity was detected in *R. meliloti*. This result is consistent with the occurrence of an aspartate utilization mutant such as 4R3, since the aspartase catabolic pathway would otherwise provide an alternate means of degrading this substrate. In comparison, a mutant with the equivalent growth defect is not known in *E. coli*, which possesses both enzymes. Poole et al. (31) have found that aspartase is also not produced in *R. leguminosarum* bv. viceae but demonstrated that aspartase activity could be acquired in their strain by mutation.

Since mutant 4R3 grows on glutamate, AspAT is not required for growth of *R. meliloti* on glutamate as a carbon source, as in *E. coli* (29). This observation is also consistent with the absence of aspartase activity in *R. meliloti*, since this enzyme would be required to return the aspartate carbon to the TCA cycle for regeneration of oxaloacetate. The process is part of a deamination cycle including AspAT, aspartase, fumarase, and malate dehydrogenase. In *R. meliloti*, an analogous deamination cycle must be required for glutamate during the reverse AspAT reaction, when aspartate is to be catabolized. When aspartate is converted to oxaloacetate by AspAT there is an obligate formation of glutamate, which must be cycled back to α-ketoglutarate. In *R. meliloti* either of two routes is possible: through glutamate dehydrogenase to α-ketoglutarate, or through glutamate decarboxylase to γ-aminobutyrate (the GABA shunt).

Many aminotransferases are known to have broad substrate activity (17, 22). It was found that in *R. meliloti* there is at least one other enzyme, an AroAT encoded within region II, which is capable of partial activity with aspartate as a substrate. Thus, 4R3 does not completely lack enzyme activity equivalent to that of AspAT, and there is sufficient aminotransferase activity to supply aspartate for protein

synthesis and the various other biosynthetic pathways for which it is required. A comparable situation exists in *E. coli*, in which it is necessary to mutate several genes encoding aminotransferases in order to derive an aspartate auxotroph (17). Although other aminotransferase activities are apparently present in *R. meliloti*, it is the major AspAT activity detectable in cell extracts which was found to be affected in 4R3.

The ability of the region II AroAT activity to complement the region I mutation can be attributed to a shift in the balance of aminotransferase activities in the cell. In the mutant cell, which contains a functional region II within the genome, the level of AroAT enzyme activity must be insufficient to process enough aspartate to oxaloacetate to permit growth on aspartate as a carbon source. However, when aspartate is the sole nitrogen source, the level of AroAT activity permits some growth, but at less than the rate of the wild type. When region II is present on a cosmid, it apparently causes an overexpression of AroAT activity sufficient to substitute for the complete catabolic functions of AspAT.

The results described here suggest that AspAT activity is required for an effective symbiosis between *R. meliloti* and alfalfa. The symbiotic defect, like the aspartate utilization defect, is corrected by overproduction of AroAT enzyme, such that it is apparent that it is the enzymatic activity itself, rather than any secondary effect of the mutation in region I, that is responsible for the phenotype.

What is the role of AspAT in the nitrogen fixation symbiosis? AspAT occupies a central position in cellular metabolism such that its absence could have unanticipated effects within the nodule. One possible explanation for the symbiotic block is that aspartate is provided as an essential substrate for the bacteria in the nodule. In support of this notion, the only phenotype which was found in free-living cells of 4R3 was the inability to grow on aspartate, and this was always accompanied by a Fix^- nodule phenotype. It is also pertinent that 4R3 has partial aminotransferase activity on aspartate, enough for lesser metabolic functions, but cannot process sufficient aspartate to sustain its carbon and energy requirements for growth. For this reason, it is unlikely that a minor AspAT biosynthetic capability is limiting the metabolism of 4R3 bacteria in the nodule.

If bacteria within the nodule obtained nitrogen from aspartate, there would be no need to assimilate nitrogen in the form of ammonia. This expectation is consistent with studies of the symbiotic effectiveness of *R. meliloti* mutants lacking the glutamine synthetase and glutamate synthase enzymes necessary for utilization of ammonia. Three glutamine synthetase activities have been identified in *R. meliloti*, but none is required for symbiotic nitrogen fixation (6, 39). Similarly, mutants lacking glutamate synthase activity have been described (27, 30). They were found to be symbiotically effective despite an inability to grow with ammonia as a nitrogen source. A model suggesting that a nitrogen-containing compound, such as an amino acid, is provided to bacteroids in the nodule has previously been proposed by Kahn et al. (24).

Previously, we have shown that *dct* mutants of *R. meliloti* cannot grow on aspartate as a carbon source, since the dicarboxylate transport system is required for its transport. The fact that *dctA* mutants are symbiotically defective could be due to their inability to transport aspartate, rather than their inability to transport succinate, fumarate, or malate. Further studies are necessary to establish the role of AspAT activity in the *R. meliloti* symbiosis and to determine which C_4-dicarboxylates are provided to bacteroids in the nodule. However, the phenotypes exhibited by mutant 4R3 are compelling evidence to further consider aspartate as an essential substrate during the symbiosis.

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