Regulation of the TCA cycle and the general amino acid permease by overflow metabolism in *Rhizobium leguminosarum*

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Mutants of *Rhizobium leguminosarum* were selected that were altered in the uptake activity of the general amino acid permease (Aap). The main class of mutant maps to *sucA* and *sucD*, which are part of a gene cluster *mdh-sucCDAB*, which codes for malate dehydrogenase (*mdh*), succinyl-CoA synthetase (*sucCD*) and components of the 2-oxoglutarate dehydrogenase complex (*sucAB*). Mutation of either *sucC* or *sucD* prevents expression of 2-oxoglutarate dehydrogenase (*sucAB*). Conversely, mutation of *sucA* or *sucB* results in much higher levels of succinyl-CoA synthetase and malate dehydrogenase activity. These results suggest that the genes *mdh-sucCDAB* may constitute an operon. *suc* mutants, unlike the wild-type, excrete large quantities of glutamate and 2-oxoglutarate. Concomitant with mutation of *sucA* or *sucD*, the intracellular concentration of glutamate but not 2-oxoglutarate was highly elevated, suggesting that 2-oxoglutarate normally feeds into the glutamate pool. Elevation of the intracellular glutamate pool appeared to be coupled to glutamate excretion as part of an overflow pathway for regulation of the TCA cycle. Amino acid uptake via the Aap of *R. leguminosarum* was strongly inhibited in the *suc* mutants, even though the transcription level of the *aap* operon was the same as the wild-type. This is consistent with previous observations that the Aap, which influences glutamate excretion in *R. leguminosarum*, has uptake inhibited when excretion occurs. Another class of mutant impaired in uptake by the Aap is mutated in polyhydroxybutyrate synthase (*phaC*). Mutants of succinyl-CoA synthetase (*sucD*) or 2-oxoglutarate dehydrogenase (*sucA*) form ineffective nodules. However, mutants of *aap*, which are unable to grow on glutamate as a carbon source in laboratory culture, show wild-type levels of nitrogen fixation. This indicates that glutamate is not an important carbon and energy source in the bacteroid. Instead glutamate synthesis, like polyhydroxybutyrate synthesis, appears to be a sink for carbon and reductant, formed when the 2-oxoglutarate dehydrogenase complex is blocked. This is in accord with previous observations that bacteroids synthesize high concentrations of glutamate. Overall the data show that the TCA cycle in *R. leguminosarum* is regulated by amino acid excretion and polyhydroxybutyrate biosynthesis which act as overflow pathways for excess carbon and reductant.

Keywords: 2-oxoglutarate dehydrogenase, malate dehydrogenase, succinyl-CoA synthetase, poly- β -hydroxybutyrate

INTRODUCTION

The legume-*Rhizobium* symbiosis requires the exchange of carbon and nitrogen between the plant and bacterial symbionts to fuel nitrogen fixation. In return for a source of fixed nitrogen as ammonia the plant must provide the bacterial partner (bacteroid) with a carbon and energy source. A substantial body of evidence indicates that the principal carbon source is a C₄-dicarboxylic acid, either L-malate, fumarate or succinate. This conclusion is based on the observation that C₄-dicarboxylates support high rates of respiration in isolated bacteroids (Glenn & Dilworth, 1981) and mutations in either malic enzyme or the structural gene for the dicarboxylate transport system (*dctA*) abolish nitrogen fixation, while mutations preventing sugar catabolism have no effect (Ronson & Primrose, 1979; Ronson *et al.*, 1981; Finan *et al.*, 1983; Glenn *et al.*, 1984; Arwas *et al.*, 1985; Bolton *et al.*, 1986; Engelke *et al.*, 1987). Furthermore, gluconeogenic enzymes are expressed in bacteroids of *Rhizobium leguminosarum*, *Sinorhizobium meliloti* and *Rhizobium* strain NGR234, indicating that significant quantities of sugars are not supplied to bacteroids by the plant (McKay *et al.*, 1985; Finan *et al.*, 1991; Osteras *et al.*, 1991). Consistent with these observations, the plant-derived peribacteroid membrane of *Bradyrhizobium japonicum*, which is relatively impermeable to sugars, has an active uptake system for dicarboxylic acids (Day & Udvardi, 1993). However, nutrient exchange may be more complex, since isolated bacteroids of *R. leguminosarum* and *B. japonicum* are known to excrete amino acids, particularly alanine and to a lesser extent aspartate (Appels & Haaker, 1991; Kouchi *et al.*, 1991; Rosendahl *et al.*, 1992). While on the one hand this has led to the proposal that a form of malate-aspartate shuttle may operate between the plant and bacteroid, the alternative explanation is that amino acids may be excreted to remove excess carbon and reducing equivalents (McDermott *et al.*, 1989; Appels & Haaker, 1991).

Labelling studies with isolated bacteroids of *B. japonicum* have shown that intracellular glutamate accumulates rapidly after addition of precursors such as malate (Salminen & Streeter, 1987). Similarly, when whole nodules of peas and soybeans were incubated in ¹⁴CO₂, bacteroids of both species accumulated large pools of glutamate and alanine (Salminen & Streeter, 1992). These studies suggest that the TCA cycle may become partially blocked at the 2-oxoglutarate dehydrogenase complex, leading to glutamate biosynthesis. It has also been shown that the 2-oxoglutarate dehydrogenase complex is very sensitive to inhibition by a high NADH/NAD⁺ ratio (Salminen & Streeter, 1990). If such a redox inhibition of the 2-oxoglutarate dehydrogenase complex occurs in the low O₂ environment of the nodule then glutamate biosynthesis may occur. Amino acid biosynthesis and excretion may thus be part of an overflow pathway that removes excess carbon and reductant when the TCA cycle becomes limited or blocked. In addition, polyhydroxybutyrate (PHB) biosynthesis may function

in a similar way in rhizobia (Bergersen & Turner, 1990; Povolito *et al.*, 1994; Cevallos *et al.*, 1996).

Given the importance of amino acid metabolism in the nodule we have been investigating amino acid transport and metabolism in *R. leguminosarum*. Studies revealed that *R. leguminosarum* has a novel general amino acid permease (Aap) able to transport a wide range of amino acids with high affinity (Poole *et al.*, 1985; Walshaw & Poole, 1996). We have recently cloned this system and shown it to be an ABC transporter composed of four genes (*aapJQMP*) that code for a complex able to promote the active uptake of a broad range of L-amino acids (Walshaw & Poole, 1996). This is unusual in that previously characterized ABC transporters of amino acids are highly specific for a single amino acid or a group of structurally similar amino acids. A further novel feature of the Aap is that it influences the efflux of intracellularly synthesized glutamate. For example, mutation of *aapJ* prevented 76% of glutamate excretion under appropriate conditions. We have proposed that either the Aap is bi-directional, allowing efflux of solute itself, or that it regulates a separate efflux channel/transporter (Walshaw & Poole, 1996).

Glutamate excretion by laboratory cultures of *R. leguminosarum* bv. *viciae* strain 3841 can be induced when they are grown on glucose/aspartate; this effect is dependent on the active unregulated accumulation of aspartate by the Dct system (Reid *et al.*, 1996). Aspartate loaded by the Dct system presumably acts as an amino group donor to 2-oxoglutarate forming glutamate, which is then excreted. Under these conditions uptake by the Aap is severely reduced even though transcription of the operon is slightly elevated (Reid *et al.*, 1996). These results suggest that the uptake activity of the Aap may be regulated by the TCA cycle via the synthesis of amino acids. In this study we demonstrate that such regulation does occur. A key enzyme is shown to be the 2-oxoglutarate dehydrogenase complex, confirming it as a pivotal step in the regulation of the TCA cycle in *R. leguminosarum*.

METHODS

Bacterial strains and culture conditions. Bacterial strains, bacteriophages and plasmids used in this study are described in Table 1. *R. leguminosarum* strains were grown at 28 °C on either TY (Beringer, 1974) or acid minimal salts (AMS) medium (pH 7.2) (Poole *et al.*, 1994b). All carbon and nitrogen sources were at 10 mM unless otherwise stated. Antibiotics were used at the following concentrations in µg ml⁻¹: kanamycin 40, streptomycin 500, tetracycline 2 (in AMS) and 5 (in TY), and gentamicin 20. *Escherichia coli* strains were grown at 37 °C on LB, with antibiotic concentrations in µg ml⁻¹ as follows: kanamycin 25, tetracycline 10, gentamicin 5 and ampicillin 50.

DNA and genetic manipulations. All routine DNA analysis was done essentially as described by Sambrook *et al.* (1989). Conjugations were done as previously described (Poole *et al.*, 1994b). Transductions were done with the bacteriophage RL38 as described by Buchanan-Wollaston (1979). Strain 3841 was mutagenized with Tn5 by conjugation with *E. coli* S17-1

Table 1. Bacterial strains, plasmids and bacteriophages used

Strain, phage or plasmid	Description	Source or reference
Bacteria		
<i>R. leguminosarum</i>		
3841	Str ^R derivative of strain 300	Johnston & Beringer (1975); Glenn <i>et al.</i> (1980)
RU116	Strain 3841 <i>sucD</i> ::Tn5	This work
RU137	Strain 3841 <i>phaC</i> ::Tn5	This work
RU156	Strain 3841 <i>sucA</i> ::Tn5	This work
RU724	pRU3067 homogenote in strain 3841, <i>sucA</i> ::Tn5	This work
RU725	pRU3059 homogenote in strain 3841, <i>sucC</i> ::Tn5	This work
RU726	pRU3069 homogenote in strain 3841, <i>sucB</i> ::Tn5	This work
RU733	pRU3061 homogenote in strain 3841, <i>sucA</i> ::Tn5	This work
<i>E. coli</i>		
803	<i>met gal</i>	Wood (1966)
S17-1	<i>pro hsdR recA</i> [RP4-2(Tc::Mu) (Km::Tn7)], RP4 integrated into its chromosome	Simon <i>et al.</i> (1983)
MC1061	<i>hsdR mcrB araD139 Δ(araABC-leu)7679 ΔlacX74 galU galK rpsL thi</i>	Meissner <i>et al.</i> (1987)
Plasmids		
pAR36A	<i>R. leguminosarum glnII::lacZ</i> translational fusion in pMP220	Patriarca <i>et al.</i> (1992)
pBluescript II SK-	Phagemid, pUC19 derivative, f1 origin of replication, ColE1 replicon, Amp ^R	Stratagene
pJQ200	pACYC derivative, P15A origin of replication, Gm ^R	Quandt & Hynes (1993)
pMP220	IncP transcriptional fusion vector, Tc ^R	Spaink <i>et al.</i> (1987)
pPHJ11	P-group chaser plasmid, Gm ^R	Hirsch & Beringer (1984)
pRU3004	pLAFR1 cosmid containing <i>mdh-sucCDAB</i> from strain 3841	This work
pRU3024	pLAFR1 cosmid containing <i>aapJQMP</i> from strain 3841	Walshaw & Poole (1996)
pRU3028	pRU3024 <i>aapJ</i> ::Tn5- <i>lacZ</i>	Walshaw & Poole (1996)
pRU3059	pRU3004 <i>sucC</i> ::Tn5- <i>lacZ</i>	This work
pRU3061	pRU3004 <i>sucA</i> ::Tn5- <i>lacZ</i>	This work
pRU3067	pRU3004 <i>sucA</i> ::Tn5- <i>lacZ</i>	This work
pRU3068	pRU3004 <i>sucA</i> ::Tn5- <i>lacZ</i>	This work
pRU3069	pRU3004 <i>sucB</i> ::Tn5- <i>lacZ</i>	This work
pRU3070	pRU3004 <i>mdh</i> ::Tn5- <i>lacZ</i>	This work
pRU3075	pRU3004 <i>sucA</i> ::Tn5- <i>lacZ</i>	This work
pRU3076	pRU3004 <i>mdh</i> ::Tn5- <i>lacZ</i>	This work
pSP72	Promoterless cloning vector, Amp ^R	Promega
pSUP202-1::Tn5	<i>mob</i> Km ^R	Simon <i>et al.</i> (1983)
Bacteriophages		
λ::Tn5- <i>lacZ</i>	λ carrying the Tn5-B20 transposon	Simon <i>et al.</i> (1989)
RL38	Generalized transducing phage of <i>R. leguminosarum</i>	Buchanan-Wollaston (1979)

containing pSUP202-1::Tn5 essentially as described by Simon *et al.* (1983). Cosmid pRU3024 was mutagenized with Tn5-*lacZ* by using λ containing the transposon derivative B20 essentially as described by Simon *et al.* (1989). To create chromosomal mutations, mutated cosmids were conjugated into *R. leguminosarum* strain 3841. After purification, the incompatible plasmid pPHJ11 was conjugated into each strain and the homogenotes isolated by the technique of Ruvkun & Ausubel (1981).

All sequencing was done by the cycle-sequencing method using a Promega fmol kit according to the manufacturer's instructions. GCG software was used for computer-assisted sequence analysis. Primers P₀ and P₁₅ have the sequences GTTCAGGACGCTACTTG and GGATCCATAATTTTTTCCTCC, respectively.

Transport assays. *R. leguminosarum* strains were prepared and transport assays done as previously described (Poole *et al.*, 1985), using in each case a total solute concentration of 25 μM. The specific activities of labelled solutes in the assays were: L-[U-¹⁴C]aspartic acid (354 MBq mmol⁻¹), L-[U-¹⁴C]glutamic acid (357 MBq mmol⁻¹), L-[U-¹⁴C]alanine (348 MBq mmol⁻¹), 2-amino[1-¹⁴C]isobutyrate (2.22 GBq mmol⁻¹), [2,3-¹⁴C]-succinic acid (4 GBq mmol⁻¹) and D-[U-¹⁴C]glucose (358 MBq mmol⁻¹).

Enzyme assays. Cultures of *R. leguminosarum* strains (500 ml) were harvested at a cell density of approximately 5 × 10⁸ cells ml⁻¹, washed and resuspended in 10 ml 40 mM HEPES pH 7.0, containing 1 mM DTT. Cells were disrupted by two passages through a French press at 69 MPa. Following

centrifugation at 30000 *g* for 20 min, the supernatant was used for enzyme assay. Citrate synthase (EC 4.1.3.7), 2-oxoglutarate dehydrogenase (EC 1.2.4.2), isocitrate dehydrogenase (EC 1.1.1.41) and succinyl-CoA synthetase (EC 6.2.1.5) were assayed according to Reeves *et al.* (1971). Malate dehydrogenase (EC 1.1.1.37) was assayed by the technique of Saroso *et al.* (1986). β -Galactosidase fusions were assayed according to Miller (1972), with modifications as described by Poole *et al.* (1994a).

Excretion assays. Cells of strains 3841, RU116 and RU156 were grown to mid-exponential phase overnight on malate/ NH_4Cl , glucose/ NH_4Cl /aspartate or glucose/ NH_4Cl minimal medium, harvested aseptically, washed once in AMS and resuspended to an OD_{600} of 1 in AMS. The same carbon and nitrogen sources were then added to each culture at 10 mM except for aspartate, which was 20 mM. Resuspended cells were incubated at 28 °C and samples removed at 0, 60, 150 and 270 min. Samples were centrifuged at 11000 *g* for 15 min to remove cells and the supernatant assayed for glutamate by the technique of Bernt & Bergmeyer (1974). Aspartate was measured by a procedure adapted from Bergmeyer *et al.* (1974). Each assay contained 150 μmol potassium phosphate (pH 7.2), 0.3 μmol NADH, 15 μmol 2-oxoglutarate, 2 U aspartate aminotransferase, 2 U malate dehydrogenase and 250 μl of sample, in a total volume of 1.5 ml. Alanine was determined in a final volume of 1.5 ml containing 75 μmol glycine, 60 μmol hydrazine monohydrate, 4 μmol NAD^+ , 0.5 U alanine dehydrogenase and 250 μl of sample. Assays for aspartate and alanine were incubated and read as for the glutamate determination.

2-Oxoglutarate assays contained 150 μmol potassium phosphate (pH 7.2), 0.3 μmol NADH, 75 μmol aspartate, 2 U aspartate aminotransferase, 2 U malate dehydrogenase and 250 μl of sample, in a total volume of 1.5 ml. Assays were incubated and read as for the glutamate determination. Oxaloacetate was measured as for 2-oxoglutarate except that aspartate and aspartate aminotransferase were omitted.

Intracellular metabolite concentrations. Cultures of *R. leguminosarum* strains (500 ml) were grown on glucose/ NH_4Cl , harvested by centrifugation at a cell density of approximately 5×10^8 cells ml^{-1} , washed three times in AMS and resuspended in 15 ml 100 mM HEPES, pH 7.2, all operations being carried out at 5 °C. Cells were disrupted by two passages through a French press at 69000 kPa, centrifuged at 30000 *g*, 5 °C, for 20 min, and the supernatant assayed for protein content. Ice-cold 10% trichloroacetic acid (2.5 ml) was added to 5 ml of supernatant. After centrifugation at 3500 *g*, 5 °C, for 15 min, the pH of the supernatant was adjusted to 7.0 and the volume made up to 8 ml. Samples of the neutralized supernatant were assayed for glutamate and 2-oxoglutarate as described above.

PHB determination. PHB content was determined in cultures (50 ml) grown on glucose/ NH_4Cl to a cell density of approximately 10^9 cells ml^{-1} , washed in AMS, pelleted by centrifugation and resuspended in technical grade sodium hypochlorite (5 ml). Tenfold and 100-fold dilutions of this were then made in sodium hypochlorite and incubated at 37 °C for 1 h. The PHB content was then determined by the technique of Law & Slepecky (1961).

Protein determination. The protein concentration of whole cells was determined by the method of Lowry, using BSA as standard.

RESULTS

Aspartate resistant mutants of *R. leguminosarum* strain 3841

We were interested in selecting mutants of *R. leguminosarum* altered in the regulation of transport via the Aap. Since uptake via the Aap is severely reduced in the presence of aspartate in the growth medium (Reid *et al.*, 1996) we considered that it might be possible to isolate regulatory mutants by selecting cells that had become resistant to high levels of aspartate. Toxicity is apparently caused by the loading of aspartate via the Dct system, with *dct* mutants being resistant to high levels of aspartate (150 mM) in the growth medium (Reid *et al.*, 1996; Walshaw & Poole, 1996). Conversely, *aap* mutants are more severely affected by the presence of aspartate in the growth medium, consistent with a role for the Aap in excretion of glutamate formed from aspartate (Walshaw & Poole, 1996).

Over 100000 Tn5 mutants of strain 3841 were plated on AMS agar containing glucose/ NH_4Cl as the carbon/nitrogen source, 100 mM aspartate and kanamycin at 80 $\mu\text{g ml}^{-1}$. Fifty colonies, from among several hundred able to grow on this medium, were purified and the uptake of aspartate and glucose by the resulting strains was investigated. Approximately half of the mutants analysed had severely impaired rates of aspartate uptake, even when grown in the absence of aspartate in the growth medium (data not shown). Whenever there was a reduction in aspartate transport there was a concomitant, though not so severe, reduction in glucose transport, a different phenotype from that resulting from a mutation of the Aap (Walshaw & Poole, 1996) suggesting that these mutants were altered in a gene (or genes) with a more global impact. Two strains from this class, RU116 and RU156, were chosen for further study. A second class of mutant, represented by strain RU137, had a reduction of approximately 60% in aspartate transport.

Amino acid transport in strains RU116 and RU115

Two acidic and two aliphatic amino acids were chosen to test uptake by the Aap. The Aap is responsible for approximately 80% of glutamate and aspartate transport and approximately 55% of uptake of the two aliphatic amino acids. The uptake of all the amino acids tested was significantly reduced in strains RU116 and RU156 relative to strain 3841 (Table 2). In glucose-grown cells of RU156 for example, aspartate transport was less than 5% of that in the wild-type strain. While glucose transport in strain RU156 was affected, it was still 30% of that in strain 3841. 2-Aminoisobutyrate (AIB) transport was also measured because this amino acid is transported by the Aap but is not metabolized in *R. leguminosarum* strain 3841. Since the rate of AIB transport was lowered in strains RU116 and RU156, it appears that this reduction is not simply the result of a reduced rate of metabolic incorporation. When strains RU116 and RU156 were grown on succinate, the

Table 2. Rates of amino acid transport in *R. leguminosarum* strains RU116 and RU156 grown on glucose/NH₄Cl and succinate/NH₄Cl

Rates of uptake are expressed as nmol min⁻¹ (mg protein)⁻¹. Values are the mean $\pm$ SEM of determinations from three or more independent cultures. AIB, 2-aminoisobutyrate. ND, Not determined.

Growth conditions and strain	Rate of uptake with substrate shown:					
	L-Aspartate	L-Glutamate	L-Alanine	AIB	D-Glucose	Succinate
Glucose/NH₄Cl						
3841	3.9 $\pm$ 0.1	5.6 $\pm$ 0.4	6.4 $\pm$ 0.7	4.3 $\pm$ 0.6	39.5 $\pm$ 3.2	ND
RU116	0.6 $\pm$ 0.1	0.8 $\pm$ 0.3	2.0 $\pm$ 0.2	1.2 $\pm$ 0.1	19.5 $\pm$ 0.6	ND
RU156	0.2 $\pm$ 0.1	0.3 $\pm$ 0.1	1.2 $\pm$ 0.1	0.1 $\pm$ 0.0	12.4 $\pm$ 1.1	ND
Succinate/NH₄Cl						
3841	3.1 $\pm$ 0.1	3.6 $\pm$ 0.4	ND	ND	ND	46.7 $\pm$ 0.2
RU116	1.3 $\pm$ 0.1	1.4 $\pm$ 0.4	ND	ND	ND	31.2 $\pm$ 0.6
RU156	0.6 $\pm$ 0.1	0.5 $\pm$ 0.1	ND	ND	ND	22.7 $\pm$ 3.9

reduction in the rates of glutamate and aspartate transport, relative to the wild-type, were less than those found in glucose-grown cultures. However, the reduction in amino acid uptake was still significantly greater than that of succinate uptake in each case. Succinate uptake in strains RU116 and RU156 grown on succinate was also less severely affected than glucose uptake in the same strains grown on glucose. This suggests that growth on succinate is able to partially rescue amino acid uptake in strains RU116 and RU156 as a result of a global effect within the cell.

Strains 3841, RU116 and RU156 had mean generation times on glucose of 270, 405 and 721 min, respectively. However, on succinate the respective mean generation times were 220, 315 and 305 min, indicating that the mutations in strains RU116 and RU156 were more deleterious to glucose catabolism than they were to that of succinate. Strain RU137 grew at a similar rate to strain 3841.

Genetic analysis of mutant strains

In order to confirm that the insertion of Tn5 was linked to the phenotype of the chosen aspartate toxic-escape mutants, the kanamycin resistance marker of each of strains RU116, RU137 and RU156 was transduced to strain 3841. Five transductants from each mutant strain were purified and each found to be resistant to 100 mM aspartate. In addition, aspartate and glucose uptake in the transductants was similar to the rates in their respective transductional donor strains (data not shown). These results show that the phenotypes of strains RU116, RU137 and RU156 are each tightly linked to a single Tn5 insertion.

Chromosomal DNA from strain RU116, RU137 and RU156 was isolated, restricted with *Eco*RI and the resulting restriction fragments randomly cloned into the *Eco*RI site of pBluescript II SK-. In each case, the

resulting DNA was used to transform *E. coli* strain MC1061, and Km^R Amp^R transformants were selected. *Bam*HI and *Hind*III sub-clones were made for each transposon-containing clone to enable the left and right arms to be separated for sequencing.

Each of the sub-clones was then partially sequenced using primer P₀, which binds to the end of IS50, yielding sequence directly in the flanking chromosomal DNA. The GenBank and EMBL databases were searched for sequences showing homology to each of the six possible translations of each sequence. One translation of the nucleotide sequence from chromosomal DNA flanking the transposon in strain RU116 had 54% identity to residues 223–289 of *sucD* from *E. coli*, which codes for the α -subunit of succinyl-CoA synthetase. One translation of the nucleotide sequence from the transposon in strain RU156 had 68% identity to *sucA* from *E. coli*, which codes for the 2-oxoglutarate dehydrogenase subunit of the 2-oxoglutarate dehydrogenase complex. These data show that strains RU116 and RU156 are mutated in genes coding for successive enzymes of the TCA cycle.

One translation of the nucleotide sequence from strain RU137 showed 61% and 81% identity over 88 amino acids to poly- β -hydroxybutyrate synthase (encoded by *phaC*) from *S. meliloti* and *R. phaseoli*, respectively (Povolo *et al.*, 1994; Cevallos *et al.*, 1996). This suggests that biosynthesis of PHB is blocked in strain RU137. To confirm this, the PHB content was measured in strains 3841 and RU137 grown on glucose/ammonia; it was 61 μ g (mg dry wt)⁻¹ and 4.1 μ g (mg dry wt)⁻¹, respectively.

If strains RU116 and RU156 are mutated in succinyl-CoA synthetase and 2-oxoglutarate dehydrogenase, respectively, they should not grow on arabinose or glutamate as the carbon source. This is because the catabolic pathway in *R. leguminosarum* for these substrates enters the TCA cycle at 2-oxoglutarate

Table 3. TCA cycle enzyme activities in glucose/NH₄Cl-grown aspartate toxic-escape Tn5 mutants of *R. leguminosarum* strain 3841

Activities are expressed as nmol min⁻¹ (mg protein)⁻¹. Values are the mean $\pm$ SEM of three or more independent determinations, except the succinyl-CoA synthetase values, which are the mean of two independent determinations. 2-ODH, 2-oxoglutarate dehydrogenase; SCS, succinyl-CoA synthetase; MDH, malate dehydrogenase; ICDH, isocitrate dehydrogenase; CS, citrate synthase. UD, Undetectable; ND, not determined.

Enzyme	Activity of strain:							
	3841	RU116	RU137	RU156	RU724	RU725	RU726	RU733
2-ODH	109 $\pm$ 10	6 $\pm$ 2	42 $\pm$ 12	UD	UD	UD	UD	UD
SCS	55	20	ND	252	ND	22	98	ND
MDH	4978 $\pm$ 833	18 830 $\pm$ 2524	3893 $\pm$ 1835	17 435 $\pm$ 1622	ND	ND	ND	ND
ICDH	864 $\pm$ 164	904 $\pm$ 94	890 $\pm$ 61	957 $\pm$ 66	ND	ND	ND	ND
CS	166 $\pm$ 26	227 $\pm$ 31	100 $\pm$ 23	123 $\pm$ 37	ND	ND	ND	ND

(Dilworth *et al.*, 1986). As expected, strains RU116 and RU156 were unable to grow on arabinose or glutamate as the sole carbon source.

TCA cycle enzyme activities in mutant strains of *R. leguminosarum*

Activities of the corresponding TCA cycle enzymes were assayed in strains 3841, RU116, RU156 and RU137 (Table 3). The complete loss of 2-oxoglutarate dehydrogenase activity was expected in RU156, since this strain is mutated in the gene encoding for the 2-oxoglutarate dehydrogenase sub-unit of the 2-oxoglutarate dehydrogenase complex. The observation that strain RU116 exhibited negligible 2-oxoglutarate dehydrogenase activity suggests that the transposon in RU116 (*sucD*) may be polar on *sucA*. It is relevant to note that in *E. coli* these genes are clustered as part of the *sucABCD* operon (Darlison *et al.*, 1984; Spencer *et al.*, 1984; Buck *et al.*, 1985). The ability of a *sucD* mutant to be polar on *sucA* suggests that they are also clustered in *R. leguminosarum* although the gene order is likely to be different from that in *E. coli*, because the direction of transcription in *E. coli* is from *sucA* towards *sucD* (Buck *et al.*, 1985). We show below that this is the case.

The mutation in *sucD* (strain RU116), which codes for the α -subunit of succinyl-CoA synthetase, reduced succinyl-CoA synthetase activity by 64% (Table 3). While this is consistent with the sequence data for the location of the transposon in strain RU116 it does suggest the possibility of a second gene product with succinyl-CoA synthetase activity. This activity was not due to endogenous organic acids in the enzyme extract as omitting succinate from the enzyme assay resulted in only 3% of the activity when succinate was added.

Interestingly, succinyl-CoA synthetase levels in strain RU156 were greatly increased in comparison to strain 3841, suggesting that mutation of *sucA*, and hence loss of 2-oxoglutarate dehydrogenase activity, elevates either the specific activity or expression of succinyl-CoA

synthetase. In addition the activity of malate dehydrogenase was also greatly increased in both RU116 and RU156. Activities of citrate synthase and isocitrate dehydrogenase were not increased in strain RU156 relative to 3841, so the effect does not occur for all TCA cycle enzymes.

The activity of 2-oxoglutarate dehydrogenase was reduced in strain RU137 to 38% of the activity measured in strain 3841 (Table 3). Malate dehydrogenase and isocitrate dehydrogenase were unaffected. This suggests that PHB biosynthesis is involved in the regulation of certain TCA cycle enzymes.

Structure of the *mdh-suc* operon

A chromosomal library of strain 3841 (as *EcoRI* fragments in pLAFR1) was conjugated from *E. coli* strain 803 into strain RU156. Cosmid pRU3004 was isolated that restored the ability of strains RU156 and RU116 to grow on glutamate as the sole carbon source. Amino acid uptake via the Aap was also restored in the complemented strains to the wild-type level (data not shown).

Southern blotting of *EcoRI* digested chromosomal DNA from each of strains 3841, RU116, RU137 and RU156 with pRU3004 demonstrated that pRU3004 contained DNA homologous to the Tn5-containing *EcoRI* fragment in both RU116 and RU156, but not to that in RU137. In other bacteria, *sucA* and *sucD* are clustered with genes encoding other TCA cycle enzymes (Miles & Guest, 1987; Nicholls *et al.*, 1990; Nishiyama *et al.*, 1991; Guest, 1992; Guest & Russell, 1992). It was therefore anticipated that several *R. leguminosarum* TCA cycle genes might be carried by pRU3004, in addition to *sucA* and *sucD*. Consequently, pRU3004 was subjected to further study, in an attempt to establish whether the effect on amino acid transport in strain 3841 was specific to the mutation of 2-oxoglutarate dehydrogenase and succinyl-CoA synthetase, or whether the TCA cycle in general was implicated.

Fig. 1. Map of pRU3004 which contains the *mdh-sucCDAB* region from *R. leguminosarum* strain 3841. Gene locations (numbered shaded areas) and orientations are based on identities to other bacterial genes as follows: 1, 40.4% and 38% to *mdh* in *Photobacterium* and *E. coli*, respectively; 2, 45% to *sucC* in *E. coli*; 3, 55% to *sucC* in *E. coli*; 4, 54% to *sucD* in *E. coli*; 5, 57% to *sucA* in *Coxiella burnetii*; 6, 63% and 58% to *sucA* in *C. burnetii* and *E. coli*, respectively; 7, 68% to *sucA* in *E. coli*; 8, 50% to *sucB* in *E. coli*. The sizes of these genes are only approximate and are based on homology to other bacteria. Tn5-*lacZ* insertions are shown as flags with active fusions filled. Cosmid derivatives of pRU3004 carrying a transposon are indicated above the flag, as are the strain numbers where the transposon is integrated into the chromosome. Tn5 insertions are shown as ∇ . Restriction sites are B, *Bam*HI; E, *Eco*RI; P, *Pst*I; S, *Sal*I; Ss, *Sst*I.

Cosmid pRU3004 was restriction mapped and subjected to saturation mutagenesis with Tn5-*lacZ* (Fig. 1). The Tn5-*lacZ*-containing *Sal*I fragments from pRU3059, pRU3061, pRU3069 and pRU3070 were each cloned into the *Sal*I site of either pJQ200 or pSP72. Partial sequencing of the insert DNA in these plasmids was carried out using a primer (P_{15}) to the 5' end of Tn5-*lacZ*, and either reverse, SK and KS primers to the pJQ200 polylinker, or the T7 primer to the pSP72 polylinker. One potential translation of each of these sequences exhibited significant identity to one of the TCA cycle enzymes 2-oxoglutarate dehydrogenase, succinyl-CoA synthetase or malate dehydrogenase from other organisms (Fig. 1). The locations of these identities within the various genes are consistent with the arrangements of *Rhizobium* genes proposed in Fig. 1.

To confirm the proposed directions of transcription for *mdh* and *sucCDAB*, β -galactosidase activities, produced in a strain 3841 background by corresponding pRU3004 mutants, were investigated. Those cosmids in which *lacZ* in the transposon was aligned with the proposed direction of transcription for the corresponding gene showed significantly higher activity than those in which *lacZ* was in the opposite orientation (data not shown) (Fig. 1).

In order to investigate directly the nature of the genes carried by pRU3004, activities of the corresponding TCA cycle enzymes were assayed in chromosomal mutants of these genes. Tn5-*lacZ* mutants of strain 3841 were generated by recombining the cosmids pRU3059, pRU3061, pRU3067 and pRU3069 into the chromosome to generate strains RU725, RU733, RU724 and RU726, respectively. Selection for the formation of homogenotes

was carried out on both TY and AMS agar with succinate as the carbon source. No homogenotes of cosmids pRU3070 or pRU3076 could be isolated, possibly because mutation of *mdh* is lethal. The enzyme activities were as expected for *sucCDAB* mutants (Table 3).

The polarity of *sucCD* mutations on *sucAB*, as measured by 2-oxoglutarate dehydrogenase activity, is explicable by the gene order *mdh-sucCDAB* if they share a common promoter. In turn the increase in succinyl-CoA synthetase and malate dehydrogenase activity in *sucAB* mutations may be caused by their presence in a single operon.

Transcription of the *aap* operon in *sucDA* mutants of strain 3841

Considering that amino acid uptake is particularly sensitive to disruption of the TCA cycle we wanted to determine how this effect is mediated. To determine whether regulation occurs at the transcriptional level, the activity of an *aapJ::lacZ* transcriptional fusion in strain 3841, RU116 and RU156 backgrounds was investigated.

Cosmid pRU3028, which has an active Tn5-*lacZ* fusion in *aapJ*, was conjugated into strains 3841, RU116 and RU156. β -Galactosidase activity was then measured after growth on a good nitrogen source (ammonia) and a poor nitrogen source (glutamate) (Table 4). The activities in strain RU116 and RU156 backgrounds were generally very similar to those in strain 3841, although activity in strain RU156 grown on glucose/glutamate was somewhat reduced, perhaps because this strain

Table 4. β -Galactosidase activities in *R. leguminosarum* strains RU116 and RU156 containing *aapJ* or *glnII* fusions

Strains were grown on AMS containing either glucose/ NH_4Cl or glucose/glutamate at 10 mM. Activities are expressed as $\text{nmol min}^{-1} (\text{mg protein})^{-1}$. Plasmids pRU3024 and pMP220 are controls for pRU3028 and pAR36A, respectively. Values are the mean $\pm$ SEM of determinations from three or more independent cultures.

Strain	Relevant genotype		β -Galactosidase activity	
	Host	Cosmid/plasmid	Glucose/ NH_4Cl	Glucose/glutamate
3841/pRU3024	Wild-type	<i>aapJ</i> QMP	40 $\pm$ 5	43 $\pm$ 1
RU116/pRU3024	<i>sucD</i> : :Tn5	<i>aapJ</i> QMP	32 $\pm$ 2	40 $\pm$ 3
RU156/pRU3024	<i>sucA</i> : :Tn5	<i>aapJ</i> QMP	41 $\pm$ 4	34 $\pm$ 4
3841/pRU3028	Wild-type	<i>aapJ</i> : :Tn5- <i>lacZ</i>	3325 $\pm$ 279	7923 $\pm$ 666
RU116/pRU3028	<i>sucD</i> : :Tn5	<i>aapJ</i> : :Tn5- <i>lacZ</i>	3225 $\pm$ 209	6484 $\pm$ 118
RU156/pRU3028	<i>sucA</i> : :Tn5	<i>aapJ</i> : :Tn5- <i>lacZ</i>	3853 $\pm$ 234	4726 $\pm$ 259
3841/pMP220	Wild-type	Promoterless <i>lacZ</i>	62 $\pm$ 3	105 $\pm$ 1
RU116/pMP220	<i>sucD</i> : :Tn5	Promoterless <i>lacZ</i>	111 $\pm$ 21	50 $\pm$ 7
RU156/pMP220	<i>sucA</i> : :Tn5	Promoterless <i>lacZ</i>	37 $\pm$ 11	40 $\pm$ 1
3841/pAR36A	Wild-type	<i>glnII</i> : <i>lacZ</i>	101 $\pm$ 14	531 $\pm$ 79
RU116/pAR36A	<i>sucD</i> : :Tn5	<i>glnII</i> : <i>lacZ</i>	270 $\pm$ 6	879 $\pm$ 66
RU156/pAR36A	<i>sucA</i> : :Tn5	<i>glnII</i> : <i>lacZ</i>	53 $\pm$ 4	513 $\pm$ 42

grew poorly under these conditions. Certainly, the activities of the fusions in strains RU116 and RU156 do not indicate a decrease in the transcription of the *aap* operon sufficient to account for the severe reduction in amino acid transport observed in strains RU116 and RU156 grown on glucose/ NH_4Cl (Table 2).

It is interesting that, like the wild-type strain, both mutants exhibited repression of the *aap* operon when grown on NH_4Cl as the nitrogen source. This implies that normal nitrogen regulation occurs in these strains. To test this further, β -galactosidase activities in strains RU116 and RU156 carrying the *R. leguminosarum* *glnII*:*lacZ* reporter fusion pAR36A (Patriarca *et al.*, 1992) were measured (Table 4). The *glnII* gene is well documented as being regulated by the *ntr* system in *R. leguminosarum* (Patriarca *et al.*, 1992). Strains RU116, RU156 and 3841 had increased β -galactosidase activity in response to a change from nitrogen-rich to nitrogen-poor conditions. Since nitrogen regulation occurs normally in the TCA cycle mutants the very low amino acid transport rate in these strains is unlikely to be due to a regulatory effect of the *ntr* system. Overall these data indicate that the very low rate of amino acid transport measured in strains RU116 and RU156 is a post-transcriptional effect.

Intracellular concentrations and excretion of 2-oxoglutarate and glutamate

Many of the effects on amino acid transport caused by disruption of the TCA cycle may result from changes in the intracellular concentration of metabolic intermediates. In particular, 2-oxoglutarate and glutamate concentrations may be affected by disruption of 2-oxoglutarate dehydrogenase activity. The intracellular

concentrations of these two compounds were therefore measured in cells grown on glucose/ammonia minimal medium. Whereas 2-oxoglutarate was undetectable in all strains, the glutamate concentrations in strains 3841, RU116 and RU156 were 4, 12 and 52 mM, respectively. Thus, disruption of succinyl-CoA synthetase activity or 2-oxoglutarate dehydrogenase activity leads to a large increase in the intracellular glutamate pool.

Since the concentration of glutamate, but surprisingly not 2-oxoglutarate, increased dramatically in strains RU116 and RU156, we tested for the possible excretion into the growth medium of these and other metabolites. Supernatants from cultures growing on L-malate/ NH_4Cl were assayed over time for alanine, aspartate, glutamate, 2-oxoglutarate and oxaloacetate (Fig. 2). L-Malate was provided as the carbon source because this is the most abundant C_4 -dicarboxylate in the nodule (Streeter, 1987), and consequently the most likely carbon source for the bacteroid. Although no excretion of alanine, aspartate or oxaloacetate was apparent, significant concentrations of glutamate and 2-oxoglutarate were detected in the supernatants from cultures of both strains RU116 and RU156. This is in contrast to the wild-type, for which no excretion of any of the four metabolites assayed was detected.

The excretion of certain metabolites, including 2-oxoglutarate, by bacteria under particular metabolic conditions is well documented (Tempest & Neijssel, 1992; Kramer, 1994; Encarnacion *et al.*, 1995), and the excretion of 2-oxoglutarate by *R. leguminosarum* strains containing mutations that block the major catabolic pathway for this substrate is not surprising. However, the excretion of glutamate by strains RU116 and RU156 suggests that a proportion of the excess 2-oxoglutarate

is converted to glutamate. The intracellular concentration data suggest that excess 2-oxoglutarate does not accumulate significantly in the mutants.

Since aspartate is a potential amino-donor in the production of glutamate from 2-oxoglutarate, we thought it possible that the escape of strains RU116 and RU156 from aspartate toxicity might be due to transamination of 2-oxoglutarate by aspartate, with subsequent excretion of the glutamate. To investigate this possibility, excretion of glutamate by strains 3841, RU116 and RU156 grown on both glucose/ NH_4Cl and glucose/ NH_4Cl /aspartate was measured (Fig. 3). It should be noted that these experiments could not be done with L-malate as a carbon source since it blocks access of aspartate to the Dct carrier (Reid *et al.*, 1996).

Excretion of glutamate by strains RU116 and RU156 was substantially increased by the presence of aspartate in the medium, consistent with increased transamination of 2-oxoglutarate, although 2-oxoglutarate excretion was still detectable (data not shown). This suggests that these strains are able to survive high levels of aspartate because the increased synthesis of intracellular 2-oxoglutarate enables aspartate to be removed by transamination.

The presence of aspartate in the medium resulted in excretion of glutamate, but not 2-oxoglutarate, by strain 3841 (data not shown). It seems likely that the excreted glutamate was derived from increased transamination of 2-oxoglutarate by aspartate. These data suggest that increased glutamate synthesis prevents a build up in the concentration of 2-oxoglutarate. The observation that 2-oxoglutarate was not excreted by 3841 grown on glucose/ NH_4Cl /aspartate implies that the rate of glutamate synthesis equals the rate of 2-oxoglutarate production. In strains RU116 and RU156 grown on glucose/ NH_4Cl or malate/ NH_4Cl , the rate of 2-oxoglutarate generation presumably exceeds the maximum rate of transamination to glutamate, and consequently 2-oxoglutarate is excreted.

Since strains RU116 and RU156 both have a high concentration of intracellular glutamate and excrete it at high rates it seemed possible that this substantial internal pool might lead to the apparent inhibition of amino acid uptake. Strains RU116 and RU156 were therefore incubated in minimal salts without carbon and nitrogen sources for up to 5 h to deplete the internal glutamate pool. No recovery in the transport rate occurred, nor was there any detectable glutamate excreted (data not shown). Since the data indicate that a large intracellular glutamate pool leads to excretion, this implies that a high intracellular glutamate concentration is not maintained in the absence of carbon and nitrogen sources over such an extended time period. The low rate of uptake by the Aap in strains RU116 and RU156 is therefore unlikely to simply be the result of inhibition of uptake by the high intracellular glutamate pool. This suggests that under conditions where there is a sustained increase in the intracellular glutamate pool there may be a mechanism for modification of the Aap that prevents

Fig. 2. Excretion of glutamate (a) and 2-oxoglutarate (b) by strains of *R. leguminosarum* grown on malate/ammonia minimal medium. 3841 (○); RU156 (△); RU116 (□). Data are given as nmol (mg cell dry wt)⁻¹, from a single representative experiment.

Fig. 3. Excretion of glutamate by strains of *R. leguminosarum* grown on glucose with either ammonia or aspartate minimal medium. ○, ●, 3841; △, ▲, RU156; □, ■, RU116. Open symbols, cells grown on glucose/ammonia/aspartate; filled symbols, cells grown on glucose/aspartate. Data are from a single representative experiment.

influx while allowing efflux. Uptake rates of solutes such as glucose, succinate and glutamate are very stable over a 5 h incubation in cells of strain 3841 resuspended in minimal salts. Thus, the failure of glutamate uptake to

recover, after incubation to deplete the internal glutamate pool, is not likely to be due to a general loss of membrane uptake in starved cells.

Plant properties

Although both strain RU116 and strain RU156 nodulated peas, the nodules were extremely small, green and clearly ineffective. Kanamycin resistant bacteria could not be recovered from nodules produced by these strains. These results are consistent with an earlier report that an undefined 2-oxoglutarate dehydrogenase mutant of *S. meliloti* formed ineffective nodules (Duncan & Fraenkel, 1979).

Since the Aap is regulated by the activity of the TCA cycle we tested the *aapJ::Tn5-lacZ* mutant strain RU543 for nodulation and acetylene reduction on peas. Of 25 nodule isolates from plants inoculated with strain RU543 all retained kanamycin resistance. Acetylene was reduced at a rate of $0.56 \pm 0.05 \mu\text{mol h}^{-1}$ per plant (mean $\pm$ SEM, $n = 3$) by strain RU543 compared to $0.45 \pm 0.02 \mu\text{mol h}^{-1}$ per plant (mean $\pm$ SEM, $n = 3$) by strain 3841, suggesting that loss of the Aap has no major effect on nitrogen fixation.

DISCUSSION

Mutation of the TCA cycle enzymes 2-oxoglutarate dehydrogenase and succinyl-CoA synthetase in *R. leguminosarum* strain 3841 leads to a severe reduction in amino acid uptake via the Aap. Uptake of other substrates such as glucose or succinate is also impaired in such mutants, probably as a result of general debilitation produced by the disruption of central metabolism, but the particularly marked reduction in amino acid transport suggests that there is a specific effect on this system.

Glutamate is excreted by *sucDA* mutants grown on both glucose/ NH_4Cl and malate/ NH_4Cl . These results are consistent with several studies which indicate that a significant proportion of malate supplied to bacteroids under nitrogen-fixing conditions is converted to glutamate (Salminen & Streeter, 1987; Kouchi *et al.*, 1991; Miller *et al.*, 1991; Salminen & Streeter, 1992). Such studies have led to the suggestion that glutamate synthesis in the bacteroid is a consequence of inhibition of 2-oxoglutarate dehydrogenase by NADH (McDermott *et al.*, 1989; Salminen & Streeter, 1990). Although complete loss of 2-oxoglutarate dehydrogenase activity is artificial, the accumulation of glutamate by free-living *sucDA* mutants appears to support this hypothesis. Examination of the intracellular concentrations and excretion rates of 2-oxoglutarate and glutamate suggest that 2-oxoglutarate feeds into the glutamate pool. A further indication of the importance of transamination in determining the fate of metabolites, and hence regulating growth of *R. leguminosarum*, is provided by the finding that addition of aspartate to the growth medium leads to an increase in glutamate excretion by *sucDA* mutants. This is consistent with an increase in transamination of 2-oxoglutarate due to

both the presence of an additional amino-donor in aspartate, and increased synthesis of 2-oxoglutarate from oxaloacetate released from aspartate. Although 2-oxoglutarate is excreted by *sucDA* mutants, there is apparently no significant increase in the intracellular concentration of this metabolite in these mutants. This is consistent with a report that radioactivity failed to accumulate in 2-oxoglutarate in bacteroids supplied with labelled malate, an effect that was ascribed to rapid conversion of 2-oxoglutarate to glutamate (Salminen & Streeter, 1990).

A reduction in 2-oxoglutarate dehydrogenase activity may be the reason for the escape from aspartate toxicity of strains RU116, RU137 and RU156. As already discussed, all of these strains have reduced 2-oxoglutarate dehydrogenase activity which should lead to increased 2-oxoglutarate being available for deamination of aspartate. This may increase removal of aspartate through the transamination of 2-oxoglutarate to glutamate by aspartate aminotransferase.

Aspartate appears to be toxic at moderately high concentrations because it is actively accumulated by the *dct* system (Reid *et al.*, 1996). Mutation of the *dct* system increases the concentration of aspartate required for toxicity from 50 to 150 mM (Walshaw & Poole, 1996). Aspartate at the moderate concentration of 10–20 mM in the growth medium has also been found to cause a severe reduction in amino acid uptake by the Aap in strain 3841. This reduction, which is dependent on the presence of the Dct system, is not due to an inhibition of transcription (Reid *et al.*, 1996; Walshaw & Poole, 1996). The effect in TCA cycle mutants reported here is similar, the difference being that strains with mutations in either *sucA* or *sucD* no longer require aspartate to be present in the medium to cause the reduction in uptake. Given that the *suc* mutant strains RU116 and RU156 grown on glucose/ammonia have large increases in the concentration of intracellular glutamate, a plausible hypothesis to explain the reduction of uptake by the Aap is that it is caused by glutamate or one of its products. This is compatible with the reduction in uptake by the Aap in strain 3841 being dependent on the presence of aspartate in the growth medium, since this presumably leads to a high intracellular concentration of glutamate.

The Aap is an ABC transporter that we have proposed is either bi-directional or activates another channel/transporter (Walshaw & Poole, 1996). Cells of strain 3841 grown on glucose/aspartate excrete glutamate, but the *aapJ* mutant strain RU543 grown on glucose/aspartate has glutamate excretion reduced by 76%, demonstrating that the Aap is involved in this process (Walshaw & Poole, 1996). It seems highly likely that the Aap is similarly required for glutamate excretion in *suc* mutants of *R. leguminosarum*. Excretion via the Aap occurs under conditions where uptake is very low, but transcription of the *aap* operon is not reduced. This suggests that the high intracellular concentration of glutamate, or a direct metabolic product of it, may mediate a post-translational modification of the Aap. Whatever the mechanism, the effect is to inhibit amino

acid uptake under conditions where the intracellular level of glutamate is very high, leading to its excretion. High levels of transcription of the *aap* operon, under conditions where uptake is suppressed, is consistent with a role for the Aap in excretion.

While the Aap has a broad specificity for amino acid uptake, mutation of it allowed the kinetic identification of an amino acid uptake system with high affinity for alanine and its non-metabolizable analogue 2-aminoisobutyrate (Walshaw & Poole, 1996). Excretion of alanine but not glutamate has been detected for bacteroids from peas and soybeans (Appels & Haaker, 1991; Kouchi *et al.*, 1991; Rosendahl *et al.*, 1992). The pyruvate dehydrogenase complex has strong structural and functional similarities to the 2-oxoglutarate dehydrogenase complex (Miles & Guest, 1987; Guest & Russell, 1992), and its inhibition by a low redox potential, as has been suggested for the 2-oxoglutarate dehydrogenase complex, might lead to the accumulation of pyruvate or its transamination product alanine. Labelling studies with pea bacteroids incubated in malate show a rapid rise in intracellular glutamate, followed by a smaller rise in alanine (Salminen & Streeter, 1992). A key question is to what extent these amino acid pools are interconverted by transamination, versus their independent formation by pathways such as alanine and glutamate dehydrogenase.

The data in this paper suggest that amino acid excretion and PHB biosynthesis are two of the principal pathways that regulate carbon and the redox balance of the TCA cycle in *R. leguminosarum*. Both pathways act as a sink for carbon and reducing equivalents such as NAD(P)H. While this has often been suggested, the work here shows they are clearly inter-regulated. These pathways become extremely important as sinks for excess NAD(P)H, which happens when the TCA cycle is inhibited. This inhibition appears to occur in the bacteroid due to a low O₂ tension and definitely occurs in TCA-cycle mutants. It seems likely that different strains and species of *Rhizobium* and *Bradyrhizobium* will partition carbon and reducing equivalents between these two and possibly other overflow pathways to varying extents, including bypass pathways such as the GABA shunt, which catabolizes glutamate, and the enzyme aspartase, which hydrolyses aspartate to fumarate and ammonia. Both of these pathways have been postulated to occur in *Rhizobium* and at least some of the enzyme activities have been demonstrated (Poole *et al.*, 1984; Jin *et al.*, 1990; Kouchi *et al.*, 1991; Fitzmaurice & O'Gara, 1993).

A long-standing question concerning the excretion of amino acids by bacteroids of *Rhizobium* is the possible participation in shuttle mechanisms with the plant, such as the malate-aspartate shuttle (Kahn *et al.*, 1985; McDermott *et al.*, 1989; Appels & Haaker, 1991). While the data in this paper do not exclude such mechanisms it is apparent that amino acid excretion occurs in *R. leguminosarum* as a normal response to limitation of the TCA cycle, independently of the plant macrosymbiont.

Furthermore, the demonstration that uptake systems such as the Aap influence efflux of amino acids, means that incubation of bacteroids or free-living cells in various amino and keto acids will lead to complex patterns of uptake and excretion. These patterns in themselves are not evidence that such cycles occur *in situ*. Excretion of a number of organic acids and amino acids has also been demonstrated in cells of *R. etli* sub-cultured several times in medium lacking thiamin and biotin (Cevallos *et al.*, 1996). The activities of thiamin-dependent enzymes such as the 2-oxoglutarate dehydrogenase complex and pyruvate dehydrogenase complex were no longer detectable after sub-culture. These results are in agreement with the effect of mutating the 2-oxoglutarate dehydrogenase complex.

The absence of any major effect on acetylene reduction in strain RU543, which is mutated in the Aap, suggests that glutamate is not an important carbon source in the bacteroid. This is supported by the absence of growth of this strain on glutamate as the carbon source in laboratory cultures (Walshaw & Poole, 1996). Unless there is a nodule-specific transport system for glutamate in the bacteroid we would not expect strain RU543 to obtain glutamate from the plant in significant quantities. This suggests that glutamate cannot be an important carbon source provided by the plant to the bacteroid. Such a conclusion is consistent with data from soybean nodules where the peribacteroid membrane is impermeable to glutamate (Udvardi *et al.*, 1990). The data presented here indicate that glutamate biosynthesis is important in overflow metabolism, not carbon nutrition, and supports the conclusions from labelling studies with pea and soybean bacteroids that glutamate is synthesized by bacteroids (Salminen & Streeter, 1987, 1992).

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Received 14 October 1996; revised 10 February 1997; accepted 19 February 1997.