Aspartate transport by the Dct system in *Rhizobium leguminosarum* negatively affects nitrogen-regulated operons

Colm J. Reid,[†] David L. Walshaw and Philip S. Poole

Author for correspondence: Philip S. Poole. Tel: +44 1734 318895. Fax: +44 1734 316671.
e-mail: p.s.poole@reading.ac.uk

School of Animal and Microbial Sciences,
University of Reading,
Whiteknights, PO Box 228,
Reading RG6 2AJ, UK

Amino acid uptake by the general amino acid permease (Aap) of *Rhizobium leguminosarum* strain 3841 was severely reduced by the presence of aspartate in the growth medium when glucose was the carbon source. The reduction in transport by the Aap appeared to be caused by inhibition of uptake and not by transcriptional repression. However, as measured with *lacZ* fusions, the Ntr-regulated gene *glnII* was repressed by aspartate. The negative regulatory effect on both the Aap and *glnII* was prevented by mutation of any component of the dicarboxylate transport (Dct) system or by the inclusion of a C₄-dicarboxylate in the growth medium, including the non-metabolizable analogue 2-methylsuccinate. As measured by total uptake and with a *dctA-lacZ* fusion, aspartate was an efficient inducer of the Dct system, but slightly less so than succinate alone or succinate and aspartate together. Thus, aspartate does not cause overexpression of DctA leading to improper regulation of other operons. Transport measurements revealed that the Dct system has an apparent K_m for succinate of 5 μ M and an apparent K_i for aspartate inhibition of succinate uptake of 5 mM. These data imply that the Dct-mediated accumulation of aspartate causes an unregulated build-up of aspartate or a metabolic product of it in the cell. This accumulation of aspartate is prevented either by mutation of the *dct* system or by the presence of a higher affinity substrate that will reduce access of aspartate to the carrier protein. Elevation or disruption of the intracellular aspartate pool is predicted to disrupt N-regulated operons and nitrogen fixation.

Keywords: aspartate transport, *dct*, *ntr*, *aap*, amino acid transport

INTRODUCTION

Nutrient exchange in legume nodules is dependent upon the micro-symbiont, or bacteroid, reducing N₂ to ammonia in return for a carbon source from the plant. Most studies indicate that the carbon source provided by the plant for use by the bacteroid is a C₄-dicarboxylic acid, either L-malate, succinate or fumarate. This conclusion is supported by the ability of C₄-dicarboxylates to allow high rates of respiration in isolated bacteroids (Glenn & Dilworth, 1981). Furthermore, 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 *et al.*, 1981; Finan *et al.*, 1983; Arwas *et al.*, 1985; Glenn *et al.*, 1984; Driscoll & Finan, 1993). The dependence of nitrogen fixation on the transport and catabolism of dicarboxylates has led to intensive study of the dicarboxylate transport (Dct) system. In *Rhizobium leguminosarum* and *Rhizobium* (now *Sinorhizobium*) *meliloti*, *dctA* codes for the putative dicarboxylate transport protein while *dctB* and *dctD* code for a two-component sensor and regulator, respectively, transcribed divergently from *dctA*, which activate transcription of *dctA* in response to the presence of dicarboxylates in the environment (Ronson *et al.*, 1984, 1987; Watson, 1990; Jiang *et al.*, 1989; Engelke *et al.*, 1989; Ronson & Astwood, 1985; Wang *et al.*, 1989). Dicarboxylates are presumably detected by DctB, either by directly binding the substrate itself or via detection of the substrate-binding state of DctA. Most models suggest that DctB

[†] **Present address:** Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DU, UK.

Abbreviation: Dct, dicarboxylate transport.

undergoes autophosphorylation and in turn phosphorylates DctD, which binds to the tandem upstream activator sites of *dctA* enabling it to activate transcription (Ronson, 1988; Yarosh *et al.*, 1989; Jording *et al.*, 1992; Ledebur *et al.*, 1990; Ledebur & Nixon, 1992).

The pattern of nutrient flux in the bacteroid appears to be more complex than a simple oxidation of C₄-dicarboxylates. Labelling studies with [¹⁴C]succinate and ¹⁴CO₂ show that in pea and soybean bacteroids there is a substantial accumulation of glutamate (Salminen & Streeter, 1987, 1992). Isolated pea bacteroids and symbiosomes, which retain the plant-derived peribacteroid membrane, have been shown to excrete both alanine and aspartate when incubated with a C₄-dicarboxylic acid (Appels & Haaker, 1991; Rosendahl *et al.*, 1992). While all these studies are in agreement that amino acids accumulate and are excreted by pea bacteroids, the significance of this is unclear. At one extreme, it has been proposed that a modified malate-aspartate shuttle is operating, which requires the co-ordinated uptake of glutamate and a C₄-dicarboxylic acid, coupled to the excretion of alanine/aspartate and 2-oxoglutarate (Appels & Haaker, 1991). An alternative explanation is that amino acids are synthesized endogenously by the bacteroid, as a mechanism to remove excess keto acids and possibly NAD(P)H. This proposal is supported by the observation that 2-oxoglutarate dehydrogenase is inhibited by a high NADH/NAD⁺ ratio in bacteroids of *Bradyrhizobium japonicum* and this is consistent with the observed accumulation of glutamate in soybean and pea bacteroids (Salminen & Streeter, 1990). In all these studies it is clear that glutamate, aspartate and alanine play a central role in bacteroid metabolism. Furthermore, it has been shown that a mutation in the gene coding for aspartate amino transferase (*aatA*) in *R. meliloti* prevents formation of effective nitrogen-fixing bacteroids (Rastogi & Watson, 1991; Watson & Rastogi, 1993). This implies that aspartate itself or transamination cycles are essential to effective nitrogen fixation by alfalfa bacteroids.

Given the importance of amino acid metabolism in bacteroids we have recently cloned and mutated the high-affinity general amino permease (*aap*) from *R. leguminosarum* (Walshaw & Poole, 1996) (EMBL X82596). The *aap* operon consists of four genes (*aapJ*, *Q*, *M*, *P*), which code for an ABC-type transport complex, which is the principal high-affinity uptake system for glutamate and aspartate. This system is unusual in that it transports a broad range of structurally distinct L-amino acids. It also has a powerful exchange activity enabling amino acids from intra- and extra-cellular pools to equilibrate. These properties suggest that this system might be crucial to any processes of amino acid cycling in bacteroids. While examining aspartate utilization, we discovered that the Aap is regulated by the level of this amino acid and we report here the nature of this effect.

METHODS

Bacterial strains and culture conditions. The strains used are described in Table 1. Bacteria were grown on either TY (Berliner, 1974) or on acid minimal salts (AMS) medium, which

is derived from that of Brown & Dilworth (1975) with the changes being: phosphate (0.5 mM), MgSO₄ (2 mM), CaCl₂ (0.17 mM) and buffering provided by MOPS (20 mM), pH 7.0. All carbon and nitrogen sources were at 10 mM. Antibiotics were used at the following concentrations (in µg ml⁻¹): kanamycin, 40; streptomycin, 500; tetracycline, 2 (in AMS), 5 (in TY); gentamicin, 20; ampicillin, 50; spectinomycin, 100; unless otherwise stated.

Transport assays. Cells were prepared and transport by the Aap assayed as previously described (Poole *et al.*, 1985), using 4.6 kBq L-[U-¹⁴C]aspartate (7.81 GBq mmol⁻¹), L-[U-¹⁴C]glutamate (9.77 GBq mmol⁻¹) or L-[U-¹⁴C]alanine (5.7 GBq mmol⁻¹) at a total substrate concentration of 25 µM. The kinetics of succinate uptake by the Dct system were measured using [2,3-¹⁴C]succinate (0.46 GBq mmol⁻¹) at various total substrate concentrations over one minute time intervals.

DNA and genetic manipulations. All routine DNA analysis was done essentially according to Sambrook *et al.* (1989). Southern blots were performed with an Amersham ECL kit according to the manufacturer's instructions. Conjugations were performed using either *Escherichia coli* strain S17-1 as the donor strain according to Simon *et al.* (1983) or as triparental matings according to Figurski & Helinski (1979) with either *E. coli* strains 803 or DH5α as the donor, and strain 803 containing pRK2013 providing the transfer functions.

Construction of a *dctA-lacZ* fusion. The cosmid pRU3001, which is derived from *R. leguminosarum* biovar *viciae* 3841 DNA (Poole *et al.*, 1994), was digested with *EcoRI* and the *dctA-B* intergenic region, previously mapped on a 0.9 kb fragment, was ligated into Bluescript II SK⁻, yielding pRU16. This was sequenced to confirm it was inserted as required. The *EcoRI* insert from pRU16 was cloned in the appropriate orientation in the *EcoRI* site of pMP220 to obtain a *dctA-p-lacZ* transcriptional promoter probe, pRU103.

***dct* insertion and deletion mutants.** These were constructed using a common scheme. Gene replacement strains were created using the suicide vector pJQ200KS as described by Quandt & Hynes (1993). All the mutants were marked by an Ω interposon encoding spectinomycin resistance, which contains transcriptional and translational stop signals in all three reading frames at either end, minimizing read-through from endogenous promoters (Prentki & Krisch, 1984). The insertions and deletions were generated in Bluescript II SK⁻, ensuring that the final clones containing the mutated allele had sufficient flanking DNA (> 700 bp at each end), for a cross-over event to occur. All these mutated *dct* alleles were cloned into the suicide vector pJQ200KS.

The starting plasmid for all gene replacements was pRU47, which contains a *HindIII* fragment from the cosmid pJ1848 spanning the complete *dct* region, cloned in Bluescript II SK⁻. This was digested with *ClaI* and religated to form pRU150. This was further digested with *XbaI* and *HindIII*, filled-in and religated to remove part of the Bluescript II SK⁻ polylinker, yielding pRU151, which was used for the construction of all subsequent mutated *dct* alleles.

The following plasmids are all pJQ200KS derivatives. Plasmid pRU324 contains an Ω interposon cloned into the *BamHI* site of *dctA* and is flanked by 1.8 kb of insert DNA on the *dctA* side and 4.2 kb on the *dctB* side. Plasmid pRU325 contains the complete *dct* region with an Ω interposon cloned into the *EcoRI* site in *dctB* and is flanked by 3.0 kb of insert DNA on the *dctA* side and 3.2 kb on the *dctB* side. Plasmid pRU168 contains *dctD* with the internal 123 bp *NruI* fragment deleted. The Ω interposon was blunt-end-ligated into this site, placing it 895 bp downstream from the start of *dctD*, and flanked by 0.73 kb of

Table 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristics	Reference
<i>R. leguminosarum</i>		
3841	Str ^R derivative of <i>R. leguminosarum</i> biovar <i>viciae</i> strain 300	Johnston & Beringer (1975)
RU543	Strain 3841 <i>aapJ</i> ::Tn5 <i>lacZ</i>	Walshaw & Poole (1996)
RU711	Strain 3841 Δ <i>dctD</i> - Ω , Str ^R Sp ^R	This work
RU714	Strain 3841 Δ <i>dctABD</i> - Ω , Str ^R Sp ^R	This work
RU727	Strain 3841 <i>dctA</i> - Ω , Str ^R Sp ^R	This work
RU730	Strain 3841 <i>dctB</i> - Ω , Str ^R Sp ^R	This work
RU865	Strain 3841 Δ <i>dctBD</i> - Ω , Str ^R Sp ^R	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)
DH5 α	<i>supE44 DlacU169</i> (ϕ 80 <i>lacZDM15</i>) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Hanahan (1983)
Plasmids		
pRK2013	ColE1 replicon with RK2 <i>tra</i> genes, helper plasmid used for mobilizing IncP and IncQ group plasmids, Km ^R	Figurski & Helinski (1979)
Bluescript II SK -	Phagemid, fl(-) origin of replication, ColE1 replicon, SK polylinker, 2.96 kb, standard cloning vector, Amp ^R	Stratagene
pMP220	IncP broad-host-range mobilizable promoter probe vector employing <i>E. coli lacZ</i> as reporter gene, 10.5 kb, Tc ^R	Spaink <i>et al.</i> (1987)
pAR36A	<i>glnIIP-lacZ</i> promoter probe cloned in pMP220, promoter derived from <i>R. leguminosarum</i> bv. <i>viciae</i> strain LPR105, Tc ^R	Patriarca <i>et al.</i> (1992)
pJQ200KS	Narrow-host-range (p15A <i>ori</i>) <i>mob sacB</i> , 5.4 kb, Gm ^R	Quandt & Hynes (1993)
pHP45 Ω	4.3 kb plasmid containing 2.0 kb Ω interposon encoding the gene for Sp ^R with transcription and translation termination signals at both ends, Amp ^R Sp ^R	Prentki & Krisch (1984)
pIJ1848	Cosmid containing <i>dctA-B-D</i> from <i>R. leguminosarum</i> strain 8002, Tc ^R	Mavridou <i>et al.</i> (1995)
pRU47	10.4 kb <i>HindIII</i> fragment containing <i>dct</i> region from pIJ1848 in Bluescript II SK -, Amp ^R	Poole <i>et al.</i> (1994)
pRU103	0.9 kb <i>dctA-B</i> intergenic region on <i>EcoRI</i> fragment from pRU16 in pMP220, <i>dctAp-lacZ</i> , Tc ^R	This work
pRU150	6.1 kb <i>ClaI</i> fragment from pRU47 religated to Bluescript II SK -, contains complete <i>dct</i> region, Amp ^R	This work
pRU151	6.1 kb <i>XbaI/HindIII</i> fragment from pRU150, filled in and religated, Amp ^R	This work
pRU168	3.6 kb <i>NotI/ApaI</i> insert from pRU157 in pJQ200KS, Δ <i>dctD</i> - Ω suicide vector, Gm ^R Sp ^R	This work
pRU193	5.5 kb <i>NotI/ApaI</i> insert from pRU401 in pJQ200KS, Δ <i>dctABD</i> - Ω suicide vector, Gm ^R Sp ^R	This work
pRU294	6.7 kb <i>NotI/ApaI</i> insert from pRU323 in pJQ200KS, Δ <i>dctBD</i> - Ω suicide vector, Gm ^R Sp ^R	This work
pRU324	8.2 kb <i>NotI/ApaI</i> insert from pRU321 in pJQ200KS, <i>dctA</i> - Ω suicide vector, Gm ^R Sp ^R	This work
pRU325	8.2 kb <i>NotI/ApaI</i> insert from pRU322 in pJQ200KS, <i>dctB</i> - Ω suicide vector, Gm ^R Sp ^R	This work
pRU3001	Cosmid from strain 3841 containing <i>dct</i> region, Tc ^R	Poole <i>et al.</i> (1994)
pRU3024	Cosmid from strain 3841 containing the <i>aap</i> operon, Tc ^R	Walshaw & Poole (1996)
pRU3028	Cosmid pRU3024 containing <i>aapJ</i> ::Tn5- <i>lacZ</i> , Tc ^R	Walshaw & Poole (1996)

insert DNA on the *dctB* side and 0.9 kb on the *dctD* side. Plasmid pRU294 contains a 1.4 kb deletion from the *EcoRI* site in *dctB* to the *SmaI* site of *dctD*; the *EcoRI* overhang was filled using the

Klenow fragment and the Ω interposon was blunt-end-cloned into this site. This deleted up to the first 164 bp of *dctD*. The Ω interposon is flanked by 3.0 kb of DNA homologous to strain

3841 on the *detA* side and 1.7 kb on the *detD* side. Plasmid pRU193 contains a deletion spanning the *det* region from the *Bam*HI site in *detA* (379 bp from the 5' end) to the *Eco*RV site in *detD* (164 bp from the 5' end), into which the Ω interposon was cloned. The Ω interposon is flanked by 1.8 kb on the *detA* side and 1.7 kb on the *detD* side. All of these clones in pBluescript II SK[−] were transferred to pJQ200KS using appropriate restriction sites in the polylinker.

Plasmids containing *det* mutations were triparentally conjugated into strain 3841 and the whole conjugation mix plated on TY containing 5% (w/v) sucrose, streptomycin and spectinomycin. This allowed direct selection for colonies containing double recombination events which appeared after 3–4 d incubation at 25 °C. These were shown to be gentamicin-sensitive, indicating loss of the plasmid and were unable to grow on succinate as the sole carbon source. Finally, isolated genomic DNA from each mutant was digested with *Hind*III and Southern-blotted with *detA*-, *detB*- and *detD*-specific plasmid probes, to confirm the expected gene replacements. The final strains produced are shown in Table 1.

RESULTS

The role of the Dct system in negatively regulating the Aap

Unlike *R. meliloti*, cells of *R. leguminosarum* strain 3841 are unable to grow on aspartate as a sole source of carbon and nitrogen. The only exception to this is when mutation and selection results in the acquisition of aspartase activity in some strains of *R. leguminosarum*, such as WU235 (Poole *et al.*, 1984). However, aspartate is capable of supporting growth of *R. leguminosarum* strain 3841 as a nitrogen source. The mean generation times were 189 min and 264 min on malate/ammonia and on malate/aspartate minimal medium, respectively. Strain 3841 grown on glucose/ammonia had a mean generation time of 270 min. This was increased to 660 min during growth on glucose/aspartate. It is particularly unusual that while aspartate causes a moderate inhibition of growth on dicarboxylates, it is profoundly inhibitory when glucose is the carbon source. Given the possible importance of aspartate in regulation of carbon metabolism in the nodule, we investigated how it affects nitrogen-regulated operons.

In *R. leguminosarum* strain 3841 aspartate uptake was low in cells grown on glucose/ammonia which is a nitrogen-excess condition (Table 2), while it was derepressed fourfold in cells grown on glucose/glutamate. This is presumably due to glutamate being a poor nitrogen source relative to ammonia, effectively causing nitrogen-limitation (Poole *et al.*, 1985, 1987). However, when cells were grown on glucose/aspartate, which was also expected to be a poor nitrogen source, aspartate uptake was even lower than that measured in cells grown on glucose/ammonia (Table 2). In all these uptake experiments the substrate was at a final concentration of 25 μ M, a concentration at which over 80% of aspartate and glutamate and 55% of alanine transport are lost in *aap* mutants (Walshaw & Poole, 1996). It is therefore possible to ascribe the effect of aspartate in the growth medium on amino acid transport specifically to the Aap.

Uptake of aspartate via the Aap in *R. leguminosarum* strain 3841 was also measured in cells grown on succinate in conjunction with ammonia, aspartate or glutamate. The rate of aspartate uptake by the Aap was low in cells grown on succinate/ammonia but high in cells grown on succinate/aspartate or succinate/glutamate (Table 2). The high rate of transport measured in succinate/aspartate-versus glucose/aspartate-grown cells suggests that the effect of aspartate is alleviated by succinate. This inhibitory/repressive effect of aspartate on the uptake of amino acids by the Aap was seen for all tested amino acids, not just aspartate itself. For example, cells of strain 3841 grown on glucose/ammonia compared to glucose/aspartate showed glutamate and alanine uptake rates reduced by 52% and 47%, respectively, by the presence of aspartate in the growth medium. This is consistent with the broad specificity of the Aap for L-amino acids.

Since succinate prevents the aspartate-dependent reduction in transport by the Aap, the Dct system might be involved. Therefore, the ability of aspartate to cause this effect was examined in a series of *R. leguminosarum* *det* mutants. Aspartate uptake in strains RU727 (*detA*), RU730 (*detB*), RU711 (Δ *detD*), RU865 (Δ *detBD*) and RU714 (Δ *detABD*) was measured after growth on glucose as the carbon source and ammonia, aspartate or glutamate as the nitrogen source (Table 2). Strains mutated in the *det* genes are unable to grow on succinate as a sole carbon source so it was not possible to measure aspartate uptake under such conditions. Aspartate uptake was also measured as a control in strain 3841 on the above substrates and also with succinate as a carbon source.

Cells of all *det* strains grown on glucose/ammonia showed normal repressed levels of uptake by the Aap due to the nitrogen-excess status of the culture. As in strain 3841, uptake of aspartate by the Aap was derepressed in all *det* strains after growth on glucose/glutamate indicating that they still respond to a poor nitrogen source. However, cells of all *det* strains grown on glucose/aspartate no longer displayed inhibition/repression of aspartate uptake by the Aap. Thus mutation of any component of the Dct system alleviates the inhibition/repression of the Aap evident in cells grown on glucose/aspartate.

Regulation of transcription from the *aapJ* and *glnII* promoters (*glnIIp*) in response to aspartate

To investigate whether the low rate of uptake in *R. leguminosarum* strain 3841 grown on glucose/aspartate is due to transcriptional repression of the *aap*, a Tn5-*lacZ* fusion to *aapJ* (Tn5-*lacZ* inserted at base 1176 of the *aap*, EMBL X82596) was used to measure transcription of the operon. When strain 3841 containing pRU3028 (pRU3024 *aapJ*::Tn5-*lacZ*) was grown on glucose/ammonia it had a β -galactosidase activity of 3325 ± 279 nmol min^{−1} (mg protein)^{−1} $\pm$ SEM. After growth on glucose/aspartate the activity was 5432 ± 770 nmol min^{−1} (mg protein)^{−1} $\pm$ SEM. The modest increase after growth on the poor nitrogen source aspartate versus the rich nitrogen source ammonia, shows that the reduction in amino acid transport after growth on glucose/aspartate does not occur at the

Table 2. Aspartate transport in *R. leguminosarum* strain 3841 and various *dct* mutants

Rates of aspartate transport (substrate concentration 25 μ M) are given in nmol min^{-1} (mg protein^{-1}) $\pm$ SEM and are the means of at least three independent cultures.

Strain	Genotype	Growth conditions*					
		G/N	G/Asp	G/Glu	S/N	S/Asp	S/Glu
3841	Wild-type	2.8 $\pm$ 0.4	1.4 $\pm$ 0.2	11.8 $\pm$ 0.4	2.1 $\pm$ 0.4	15.2 $\pm$ 0.3	10.1 $\pm$ 1.5
RU727	<i>dctA::</i> Ω	3.6 $\pm$ 0.1	8.2 $\pm$ 0.4	9.1 $\pm$ 0.4			
RU730	<i>dctB::</i> Ω	2.6 $\pm$ 0.5	7.9 $\pm$ 1.9	11.2 $\pm$ 0.6			
RU711	<i>dctD::</i> Ω	4.4 $\pm$ 0.9	11.6 $\pm$ 0.4	11.6 $\pm$ 1.0			
RU865	Δ <i>dctBD::</i> Ω	3.3 $\pm$ 0.7	17.6 $\pm$ 0.9	18.1 $\pm$ 0.8			
RU714	Δ <i>dctABD::</i> Ω	3.9 $\pm$ 1.2	15.0 $\pm$ 4.0	11.2 $\pm$ 1.0			

* G, glucose; S, succinate; N, ammonia; Glu, glutamate; Asp, aspartate.

Table 3. β -Galactosidase activities in *R. leguminosarum* strains containing *glnIIp* and *dctAp lacZ* fusions

Results are shown as ONPG hydrolysed in nmol min^{-1} (mg protein^{-1}) $\pm$ SEM from at least three independent cultures.

Strain	Genotype*	Growth conditions†									
		G/N	G/Asp	G/Glu	S/N	S/Asp	S/Glu	G/S/N	G/S/Asp	G/S/Glu	G/Asp/N
RU622	Wild-type	288 $\pm$ 26	362 $\pm$ 52	1126 $\pm$ 31	474 $\pm$ 100	1582 $\pm$ 157	1400 $\pm$ 80	441 $\pm$ 9	1616 $\pm$ 158	1587 $\pm$ 93	349 $\pm$ 39
RU782	<i>dctA::</i> Ω	379 $\pm$ 16	1293 $\pm$ 77	1407 $\pm$ 46				502 $\pm$ 23	1860 $\pm$ 102	1777 $\pm$ 135	445 $\pm$ 16
RU788	<i>dctB::</i> Ω	365 $\pm$ 34	1383 $\pm$ 110	1329 $\pm$ 60				475 $\pm$ 5	1549 $\pm$ 94	1667 $\pm$ 37	466 $\pm$ 13
RU793	<i>dctD::</i> Ω	424 $\pm$ 28	1516 $\pm$ 144	1370 $\pm$ 55				459 $\pm$ 15	1453 $\pm$ 111	1481 $\pm$ 149	445 $\pm$ 26
RU948	Δ <i>dctBD::</i> Ω	419 $\pm$ 4	1234 $\pm$ 94	1293 $\pm$ 82				363 $\pm$ 10	1603 $\pm$ 46	1580 $\pm$ 31	429 $\pm$ 11
RU802	Δ <i>dctABD::</i> Ω	403 $\pm$ 46	1252 $\pm$ 51	1286 $\pm$ 36				484 $\pm$ 49	1955 $\pm$ 50	1716 $\pm$ 145	458 $\pm$ 37
RU364	Wild-type	266 $\pm$ 61	1533 $\pm$ 356		2067 $\pm$ 267	3433 $\pm$ 101					

* All strains contain *glnII::lacZ* fusions except RU364 which contains a *dctA::lacZ* fusion.

† Abbreviations are as for Table 2.

transcriptional level. Strain 3841 containing the parent cosmid had no detectable β -galactosidase activity under these growth conditions.

To determine whether aspartate specifically reduces transport by the Aap, other nitrogen-regulated genes were investigated. One such gene in *R. leguminosarum*, whose transcription is NtrC-dependent, is *glnII* (Patriarca *et al.*, 1992; Morett *et al.*, 1985). Transcription from the *glnIIp* was therefore assayed using the *glnII-lacZ* fusion contained on pAR36A (Patriarca *et al.*, 1992). This was conjugated into strain 3841, generating strain RU622, which was grown under identical conditions as for amino acid transport and the activity of β -galactosidase measured.

Strain RU622 displayed a similar regulatory pattern for transcription from the *glnIIp* as seen for total amino acid uptake by the Aap (Table 3). Transcription from the *glnIIp* was repressed on glucose/ammonia and succinate/ammonia, which are nitrogen-excess conditions. When strain RU622 was grown on glucose/glutamate, succi-

nate/aspartate or succinate/glutamate which are nitrogen-poor conditions, transcription from the *glnIIp* was derepressed indicating that it responds to the nitrogen status of the cell. However, when strain 3841 was grown on glucose/aspartate, repression of transcription from the *glnIIp* was evident. The level of transcription was similar to that of cells grown on glucose/ammonia. Expression from the *glnIIp* in the wild-type therefore shows a comparable pattern of regulation to that for the inhibition of uptake of amino acids by the Aap. Clearly though there must be differences in the mechanism of negative regulation of *aap* and *glnII* since only the latter involves transcriptional repression. Once again, succinate appeared to be capable of alleviating the repression of the *glnIIp* caused by aspartate.

Cells were next grown on combinations of substrates to further investigate how succinate alleviates the aspartate-dependent repression. When strain RU622 was grown on glucose/succinate/aspartate, transcription from the *glnIIp* was derepressed indicating that succinate relieves the repression and that it is not caused by glucose (Table 3).

All other combinations of growth substrates gave results consistent with the nitrogen status of the medium.

Regulation of transcription from the *glnIIp* in various *dct* strains in response to the nitrogen source

Mutation of any component of the Dct system alleviated the aspartate-dependent inhibition of uptake of amino acids by the Aap after growth on glucose/aspartate. Since the pattern of repression of the *glnIIp* in cells grown on glucose/aspartate appeared to match the inhibition of uptake by the Aap, the effect of mutations in the *dct* system on transcription of the *glnIIp* were examined.

Plasmid pAR36A (*glnIIp* reporter probe) was conjugated into strains RU727 (*dctA*), RU730 (*dctB*), RU711 (Δ *dctD*), RU865 (Δ *dctBD*) and RU714 (Δ *dctABD*) generating respectively strains RU782, RU788, RU793, RU948 and RU802. These strains were grown under identical conditions as for measurement of amino acid uptake; however because they are unable to use succinate as a sole carbon source it was supplied in conjunction with glucose.

When grown under nitrogen-excess conditions (e.g. glucose/ammonia) all of the *dct* strains showed repressed levels of transcription from the *glnIIp*, while after growth under nitrogen-poor conditions (e.g. glucose/glutamate), transcription from the *glnIIp* was derepressed (Table 3). When grown on glucose/aspartate, transcription from the *glnIIp* was derepressed, which is comparable to the result obtained for uptake of amino acids by the Aap in *dct* strains. This indicates that aspartate transport mediated by the Dct system leads to negative regulation of both uptake by the Aap and *glnII* transcription.

Relief of the aspartate-dependent repression by 2-methylsuccinate

2-Methylsuccinate is an analogue of succinate which does not support growth of *R. leguminosarum* biovar *viciae* strain 3841 when supplied as the carbon source, nor does it induce the *dct* system. However, it can compete with succinate for transport, indicating it must bind to and is probably transported by DctA (Glenn *et al.*, 1980). As succinate is capable of alleviating the repression effect evident in cells grown on glucose/aspartate, we decided to investigate if 2-methylsuccinate could function in a similar fashion.

Transcription from the *glnIIp* was measured in cells grown on glucose/aspartate (repressive conditions) with different amounts of 2-methylsuccinate added. In strain RU622, transcription from the *glnIIp* was derepressed in the presence of 5 and 10 mM 2-methylsuccinate, but was repressed in the presence of 0.5 mM 2-methylsuccinate (Fig. 1). This indicates that 2-methylsuccinate alleviates the repression in a concentration-dependent fashion. This derepressed level of transcription was in contrast to the control (strain RU622 grown on glucose/aspartate alone) and was comparable to the levels of transcription observed in the wild-type (strain RU622) grown on glucose/

Fig. 1. Effect of 2-methylsuccinate in the growth medium on the activity of a *glnII-lacZ* fusion in *R. leguminosarum* strain 3841. Cultures were grown on glucose/aspartate (●) or succinate/aspartate (■) with 2-methylsuccinate at the indicated concentrations. Values shown are the means of three independent cultures $\pm$ SEM.

glutamate (nitrogen-poor conditions). The effect of 2-methylsuccinate on transcription from the *glnIIp* was also investigated in cells grown on succinate/aspartate (Fig. 1). Under these conditions it had no effect on transcription, which was always derepressed.

Uptake of aspartate by the Aap in strain 3841 was also measured in cells grown on glucose/aspartate in conjunction with 10 mM 2-methylsuccinate. The level of transport was high [11.0 ± 2.0 nmol min⁻¹ (mg protein)⁻¹ $\pm$ SEM], in contrast to the wild-type grown on glucose/aspartate alone [1.4 ± 2.0 nmol min⁻¹ (mg protein)⁻¹ $\pm$ SEM], and was similar to that observed on glucose/glutamate (nitrogen-poor conditions).

Thus, 2-methylsuccinate is capable of alleviating the inhibition of uptake of amino acids by the Aap and transcription from the *glnIIp* evident in cells grown on glucose/aspartate. This indicates that metabolism of succinate is not required for relief of the aspartate-dependent inhibition/repression. Succinate and 2-methylsuccinate presumably accomplish this by inhibiting binding of aspartate to DctA, hence lowering its intra-cellular accumulation.

Kinetics of aspartate transport in strain 3841

Since these data show that aspartate transport mediated by the Dct system causes a severe repression or inhibition of nitrogen-regulated operons, the kinetics of the Dct system for succinate uptake and inhibition by aspartate were determined. The apparent K_m for succinate uptake via the Dct system by strain 3841 was determined in cells grown on succinate/ammonia and was 5 μ M with an apparent

Fig. 2. Inhibition of succinate uptake by aspartate in *R. leguminosarum* strain 3841. Cultures were grown on succinate/ammonia. ●, Control (succinate alone); ■, succinate plus aspartate (5 mM); ▲, succinate plus aspartate (20 mM). Values shown are the means of three independent cultures $\pm$ SEM.

$V_{\max}$ of $64 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$ (Fig. 2). Aspartate is a competitive inhibitor of the Dct system with an apparent K_i of 5 mM, demonstrating it has a low affinity for binding. This indicates that at equimolar concentrations succinate would largely prevent binding of aspartate to the Dct system.

Expression level of DctA

Aspartate is a very efficient inducer of the *dct* system and the Dct-mediated effect of aspartate might result from overexpression of the transport protein relative to that which occurs after induction by succinate (Watson *et al.*, 1993; Poole *et al.*, 1994). This was investigated by conjugating the *dctA-lacZ* fusion contained on pRU103 into *R. leguminosarum* strain 3841, generating strain RU364. The induction caused by aspartate (10 mM) was 5.8-fold, while that by succinate (10 mM) was 7.8-fold (Table 3). Furthermore, succinate and aspartate together, both at 10 mM, caused an induction of 13-fold. Clearly, aspartate is not a better inducer than succinate and the inclusion of both compounds in the growth medium, which prevents the aspartate-dependent repression of *glnII*, increased the induction of *dctA* further.

DISCUSSION

It is evident that transport by the Aap is decreased when the wild-type strain of *R. leguminosarum* is grown in the presence of aspartate and a carbon source such as glucose. This is not caused by repression of transcription suggesting the effect results from inhibition of uptake by the Aap. However, transcription from the *glnIIp* was decreased by the growth on glucose/aspartate. Both effects can be relieved in two ways; firstly by the inclusion

of succinate or a non-metabolizable analogue of it in the growth medium, and secondly by mutation of any component of the Dct system.

A number of transposon mutations in the *dct* system have been found to have complex regulatory effects on other operons including *nif* and *nod* genes (Mavridou *et al.*, 1995; Birkenhead *et al.*, 1990). These have usually been found to map to *dctB* and imply that disturbance of the balance between components of the *dct* system causes improper heterologous regulation of other operons. Given that DctD is a transcriptional activator that is closely related to NtrC and NifA, it is possible that either improper expression or changes in its phosphorylation state may affect heterologous operons. In *R. meliloti*, aspartate is a very efficient inducer of the *dct* system with growth on aspartate inducing higher rates of aspartate transport than growth on succinate or malate (Watson *et al.*, 1993). This suggests that in *R. leguminosarum* strain 3841, aspartate might cause greater induction of *dctA* than substrates such as succinate, leading to improper cross-talk with other operons. By using a *lacZ* fusion to *dctA*, it was shown firstly, that aspartate does not lead to higher induction of *dctA* than that caused by succinate (Table 3), and secondly that the inclusion of succinate and aspartate together in the growth medium leads to the highest levels of *dctA* induction measured, although this combination of growth substrates prevents the aspartate-dependent repression of *glnII* and inhibition of uptake by the Aap.

A common factor linking *dctA*, *dctB* and *dctD* strains is that they all lack DctA, implying that it is the carrier protein itself that mediates aspartate repression. It is unlikely that the effect is caused by improper phosphorylation of DctB or DctD since *dctA* strains, which escape the aspartate-dependent repression, are still capable of very high rates of phosphorylation (Yarosh *et al.*, 1989; Jording *et al.*, 1992). The requirement for DctA implies that the transport of aspartate by the permease could lead to high levels of intracellular aspartate which might cause inhibition of operons such as *aapJQMP* and repression of others such as *glnII*. To investigate this, the kinetics of succinate uptake were measured in the wild-type. The *dct* system is induced by the presence of dicarboxylates including aspartate and succinate, and has an apparent K_m for succinate uptake of 5 μM , with an apparent K_i for inhibition of succinate uptake by aspartate of 5 mM. Since the expression of DctA is probably not subject to nitrogen-mediated regulation by the Ntr system, induction of *dctA* by aspartate has the potential to lead to the unregulated accumulation of intracellular aspartate. Unregulated accumulation of aspartate via DctA would lead to it, and subsequently metabolites derived from it, having a high intracellular concentration, which might repress transcription of some nitrogen-regulated operons such as *glnII* and inhibit the products of others such as the Aap.

High rates of amino acid uptake by the Aap and transcription of *glnII* were evident when either succinate or 2-methylsuccinate was present in the growth medium in conjunction with aspartate. Given the relative affinities of succinate and aspartate for DctA, when present in equal

concentrations, succinate would essentially block transport of aspartate by DctA and prevent the build-up of its intracellular concentration. 2-Methylsuccinate might operate in a similar fashion to succinate in out-competing aspartate for transport via DctA.

In a strain mutated in any component of the *dct* operon, the DctA permease would either not be transcribed or functional and the levels of intracellular aspartate would not be augmented. As a consequence, *dct* strains grown on glucose/aspartate would have moderate levels of intracellular aspartate allowing both high rates of transport by the Aap and derepression of *glnII*.

Nitrogen levels in *R. leguminosarum* are thought to be sensed by a similar mechanism to that operating in *E. coli*, where the ratio of 2-oxoglutarate to glutamine is sensed by the uridylyltransferase/uridylylremoving enzyme, resulting in changes in the uridylylation state of P_{II}. In turn, P_{II} alters the ability of NtrB to act as a kinase/phosphatase on NtrC. During nitrogen-limitation, the ratio of 2-oxoglutarate to glutamine is high, resulting in an increased level of phosphorylated NtrC, which activates the transcription of appropriate nitrogen-regulated operons (Amar *et al.*, 1994; Patriarca *et al.*, 1993). For example, *R. leguminosarum* biovar *viciae* grown under nitrogen limitation is derepressed for amino acid transport by the Aap and transcription of *glnII* (Poole *et al.*, 1985; Patriarca *et al.*, 1992).

Cellular glutamine, which is synthesized directly from glutamate by glutamine synthetase, is critical to nitrogen-sensing. Aspartate is readily converted to glutamate by aspartate aminotransferase and this increase in glutamate could lead to an increase in the intracellular concentration of glutamine, assuming glutamate is the limiting substrate for glutamine synthetase. Therefore, high levels of aspartate could result in a build-up of glutamine, which would increase the glutamine:2-oxoglutarate ratio indicative of nitrogen excess. As a consequence, this could lead to repression of Ntr-regulated operons such as *glnII*. This model may be rather simplistic though since the Aap appears to be inhibited rather than repressed by the presence of aspartate in the growth medium. Such a model implies that intracellular glutamate levels may be important. However, growth of cells of strain 3841 on glucose/glutamate with glutamate at concentrations from 10 to 50 mM did not cause inhibition of the Aap. Furthermore, strain 3841 can be grown on glutamate as the sole carbon and nitrogen source without inhibition of the Aap. Indeed, mutation of *aap* genes prevents growth on glutamate as the sole carbon source (Walshaw & Poole, 1996). However, these results can be misleading because it is the steady-state intracellular concentration of glutamate that is important. Growth on high external concentrations of amino acids may not lead to highly elevated intracellular concentrations if transport and metabolism are tightly regulated. Aspartate probably only leads to an elevated intracellular amino acid pool in strain 3841 because the Dct system is unlikely to be regulated by this pool. Regulation of amino acid transport via the Aap by inhibition, rather than by repression, may be a response to the size of the amino acid pool.

Alternatively, aspartate or a metabolic product of it could be important and changes in its intracellular concentration could signal directly to the Ntr or to other systems in the cell which are regulated by amino acid availability. For example, leucine-responsive protein is clearly involved in the regulation of amino acid uptake by a number of transport systems (Haney *et al.*, 1992; Haney & Oxender, 1992). However, the complexity of the possible metabolic fates for aspartate prevent any simple conclusion.

Aspartate metabolism is clearly very important to nitrogen signalling in *R. leguminosarum*. It is therefore interesting that aspartate aminotransferase mutants of *R. meliloti* are unable to fix nitrogen on alfalfa plants (Rastogi & Watson, 1991; Watson & Rastogi, 1993). The data in this work imply that minor perturbations in the intracellular concentration of aspartate may have quite dramatic effects on nitrogen signalling, metabolism and growth generally. This alone may explain the inability of bacteroids mutated in aspartate aminotransferase to fix nitrogen, without the need to postulate that bacteroid metabolism involves a complex malate/aspartate shuttle. However, aspartate metabolism is very different between *R. meliloti* and *R. leguminosarum*, with the former, but not the latter, able to grow on aspartate as a sole carbon and nitrogen source. *R. meliloti* catabolizes aspartate via aspartate aminotransferase, leading to the synthesis of glutamate. If the subsequent breakdown of glutamate via glutamate dehydrogenase and/or the γ -aminobutyric acid shunt, which are postulated to operate in *R. meliloti* (Fitzmaurice & O'Gara, 1991, 1993), is sufficient to prevent amino acid accumulation via aspartate aminotransferase then there should be no disruption of nitrogen regulation. Aspartate aminotransferase mutations will of course alter this balance. It has recently been shown in *Rhizobium etli* that the enzyme aspartase is normally induced by asparagine, but not aspartate, explaining its ability to grow on the former but not the latter as a carbon source (Huerta-Zepeda *et al.*, 1996). In *R. leguminosarum* only strains such as WU235, which have acquired a mutation enabling the constitutive expression of aspartase activity, can grow on aspartate as the sole carbon source (Poole *et al.*, 1984). Aspartase splits aspartate directly to fumarate and ammonia, preventing build-up of aspartate and glutamate in the cell. Since transport of aspartate is adequate to support growth in strains such as 3841 and WU235, the inability to grow in the absence of aspartase may result from imbalances in the intracellular amino acid pool. The key point is that all steps in the transport and metabolism of aspartate must operate co-ordinately and at sufficient rates to prevent the build-up of inhibitory concentrations of metabolic intermediates. Failure to do this may prevent growth on aspartate as a carbon source or nitrogen fixation by bacteroids.

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