Rhizobium leguminosarum* nodulation gene (*nod*) expression is lowered by an allele-specific mutation in the dicarboxylate transport gene *dctB

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To identify host genes that might influence *nod* (nodulation) gene expression in *Rhizobium leguminosarum*, a *nodC-phoA* reporter plasmid (carrying *nodD*) was introduced into a chemically mutagenized population of a *R. leguminosarum* strain lacking a symbiotic plasmid. The transconjugants were screened for expression of alkaline phosphatase (PhoA) on plates containing hesperetin, an inducer of *nod* genes, and a mutant with reduced expression was identified. When the *nodC-phoA* plasmid was cured from the mutant and the symbiotic plasmid pRL1J1 introduced, the mutant formed nodules, but symbiotic nitrogen fixation was less than 20% of normal. When the *nodC-phoA* allele was introduced on pRL1J1 a low level of *nod* gene induction was found. The reduced *nodC* expression appeared to be caused by a decrease in expression of the regulatory gene *nodD*, since expression of a *nodD-lacZ* fusion was also lower in the mutant than in the control. These mutant phenotypes and the low nitrogen fixation were complemented with a plasmid (pIJ1848) from a *R. leguminosarum* cosmid library. DNA hybridization confirmed that pIJ1848 was not from the symbiotic plasmid and showed that a DNA insertion was present in the mutant. The complementing region of pIJ1848 was defined by transposon mutagenesis; DNA sequencing revealed that it carried the dicarboxylic acid transport (*dct*) genes. However, the mutant grew well with succinate as sole C-source. Genetic analysis revealed that the mutant appeared to contain IS50 in the regulatory gene *dctB* and that this mutation caused the reduction in *nod* gene expression. The effect was allele-specific since other mutations in *dctB* did not influence *nod* gene expression. Surprisingly, the mutant had a constitutive high level of succinate transport, indicating that the mutation caused unregulated expression of *dctA* the structural gene for dicarboxylic acid transport. This in some way appears to have lowered the expression of *nodD*, indicating that the *nodD* promoter may be influenced by the metabolic status of the cells or by expression of *dctD* in the absence of *dctB*.

Keywords: *Rhizobium leguminosarum*, *nod*, gene regulation, succinate, symbiotic nitrogen fixation

INTRODUCTION

The nodulation (*nod*) genes of *Rhizobium leguminosarum* biovar *viciae* are involved in the synthesis of signals required for the formation of nitrogen-fixing nodules on

legumes such as *Pisum sativum* (pea) and the vetch *Vicia hirsuta* (Göttfert, 1993). In one well-characterized strain of *R. l. viciae*, 13 *nod* genes have been identified (Downie, 1991). Twelve of these, in four operons (*nodABCIIJ*, *nodFEL*, *nodMNT* and *nodO*), are under the regulatory

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Abbreviations: PhoA, alkaline phosphatase; X-P, 5-bromo-4-chloro-3-indolyl phosphate.

control of NodD (Schlaman *et al.*, 1992), a DNA-binding protein (Hong *et al.*, 1987) encoded by *nodD* in a fifth operon.

Under normal laboratory growth conditions, *nodD* is expressed constitutively (Rossen *et al.*, 1985) but the other *nod* gene operons are not induced. Specific flavonoid molecules such as naringenin, hesperetin and eriodictiol, normally present in the rhizosphere of legumes, strongly induce *nod* gene expression (Firmin *et al.*, 1986; Recourt *et al.*, 1991), and this induction is *nodD*-dependent (Rossen *et al.*, 1985; Spaink *et al.*, 1987). Circumstantial evidence indicates that there is a direct interaction between the flavonoids and NodD (Burn *et al.*, 1987; Spaink *et al.*, 1989), although no direct binding has been shown.

Several other factors can influence *nod* gene expression. It appears that the level of flavonoid-dependent induction is growth-phase dependent (Djordjevic *et al.*, 1987). In the closely related species *R. meliloti*, NH_4^+ ions can repress *nod* gene induction (Dusha & Kondorosi, 1993), although this is not the case with *R. l. viciae* (Baev *et al.*, 1992). A repressor of *nod* gene expression is found in some strains of *R. meliloti* (Kondorosi *et al.*, 1989), but a similar repressor has not been found in *R. l. viciae*. It is also evident that *nod* gene expression ceases when rhizobia become nitrogen-fixing bacteroids within plant cells; this is thought to be due to specific repression of *nod* gene expression (Schlaman *et al.*, 1991).

We took a genetic approach to identify factors that might influence flavonoid-dependent *nod* gene induction in *R. l. viciae*. A mutation that caused lowered *nod* gene expression was identified and shown to have resulted from a mutation in the *dctB* (dicarboxylate) gene.

METHODS

Bacterial strains and growth. Strains and plasmids are described in Table 1 and/or the text. Rhizobial strains were grown in TY complete medium (Berlinger, 1974), or Y minimal medium supplemented with 10 mM sodium glutamate, sodium aspartate or NH_4Cl as N-source, and 10 mM succinate, mannitol or glucose as a C-source. *Escherichia coli* was grown in LB medium (Maniatis *et al.*, 1982). Where appropriate, antibiotics were added as follows: streptomycin, 400 $\mu\text{g ml}^{-1}$; kanamycin, 20 $\mu\text{g ml}^{-1}$; tetracycline, 5 $\mu\text{g ml}^{-1}$; gentamicin, 5 $\mu\text{g ml}^{-1}$; carbenicillin, 10 $\mu\text{g ml}^{-1}$. Bacterial growth was monitored at 600 nm using a Bausch & Lomb Spectronic 20 spectrophotometer. Levels of induction of *nod* gene fusions were assessed on Y plates containing 1 μM hesperetin and 40 $\mu\text{g ml}^{-1}$ of either X-Gal or 5-bromo-4-chloro-3-indolyl phosphate (X-P) for β -galactosidase or alkaline phosphatase (PhoA) fusions, respectively. *nod* gene expression was assayed using rhizobia grown in Y medium containing mannitol and NH_4Cl and assayed for β -galactosidase (Rossen *et al.*, 1985) or alkaline phosphatase activities (Cubo *et al.*, 1992).

For succinate uptake, cells were grown to mid-exponential phase (about 5×10^8 cells ml^{-1}) in Y medium containing glucose and NH_4Cl or sodium aspartate. The cells were harvested by centrifugation, then washed and resuspended in minimal medium lacking a C- or N-source. Transport was measured as described by Poole *et al.* (1985) using 18.5 kBq of [2,3- ^{14}C]succinate (4.0 GBq mmol^{-1}) at a total substrate concentration of 25 μM .

Genetic manipulations. Plasmids were transferred by conjugation as described by Johnston *et al.* (1978). Transfer of plasmids from *E. coli* to *R. leguminosarum* involved triparental matings using the helper plasmid pRK2013 (Figurski & Helinski, 1979). Mutagenesis of pIJ1848 with Tn5-*lacZ* was done by transforming *E. coli* A118 with pIJ1848 and then selecting mutated derivatives following conjugation into *R. leguminosarum* A150, essentially as described by Surin *et al.* (1990). Transductions were done using the *R. leguminosarum* phage RL38 as described by Buchanan-Wollaston (1979).

Mutagenesis with NTG was done by placing a few crystals onto the centre of a TY plate that had been spread with a lawn of strain 8401. After 4 d at 28 °C, slowly growing cells from the zone of inhibited growth were scraped off the agar and resuspended in TY medium. After 16 h growth at 28 °C, pIJ1687 was transferred into the population by a triparental mating. Transconjugants were selected on Y medium using streptomycin and kanamycin with hesperitin plus X-P to identify mutants with altered *nod* gene expression.

The cosmid library of *R. leguminosarum* DNA in *E. coli* was that described by Lamb *et al.* (1985). The library was transferred *en masse* to *R. leguminosarum*, selecting for transconjugants on Y medium containing tetracycline, streptomycin and kanamycin, as well as hesperetin plus X-P to identify clones with increased levels of *nodC-phoA* expression in strain A131.

Mutagenesis of cosmid clones was carried out as described by Surin *et al.* (1990) using *E. coli* strain A118 and selecting for Tn5-*lacZ* insertions following conjugation into strain 8401. The Tn5-*lacZ* alleles were recombined from plasmids into the *R. leguminosarum* chromosomal *dct* genes by homologous recombination as described by Ruvkun & Ausubel (1981); recombinants were checked for the appropriate pattern of fragments by DNA hybridizations.

Transposon mutagenesis of strain A146 was carried out using the suicide plasmid pSUP102-Gm carrying Tn5-*lacZ* as described by Simon *et al.* (1989), selecting for transconjugants on TY medium containing kanamycin and streptomycin. Single colonies were then screened for growth on Y medium containing succinate plus NH_4Cl . The mutations in the *dct* region were recombined onto pIJ1848 by preparing plasmid DNA and transforming *E. coli* to kanamycin and tetracycline resistance.

DNA manipulations. Restriction enzyme digests, and ligations and subclonings were done following the methods of Maniatis *et al.* (1982). Plasmid pIJ1885 was made by digesting pIJ1848 with *Hind*III and re-ligating the DNA and screening for DNA fragments present. Only a 10 kb *Hind*III fragment was released by *Hind*III digestion of pIJ1885. This plasmid was digested with *Acc*I, and a 4.4 kb fragment was subcloned in either orientation into phagemid Bluescript SK (+) (Stratagene) to form pIJ1933 and pIJ1934.

PCR amplification of DNA from pIJ1991 and A146 was done using the oligonucleotide 5' GGAGGTCACATGGAAGT-CAG, which is homologous to nucleotides 46–65 from the end of Tn5, and the oligonucleotide 5' CGGCACCCGTCCTA-CGTCG, which is homologous to nucleotides 759–778 of the *dctB* region as described by Ronson *et al.* (1987). Following an initial denaturation for 5 min at 93 °C, conditions were 34 cycles of 45 s at 93 °C, 45 s at 58 °C, and then 2.5 min at 72 °C. The 30 μl reaction mixture contained 20 mM Tris/HCl, pH 8.0, 3 mM MgCl_2 , 0.2 mM each of dATP, dCTP, dGTP and dTTP, 0.025% (v/v) Tween Nonidet and 1 μl Taq polymerase.

DNA sequencing was done with a Pharmacia Automated Low Fluorescent DNA Sequencer using single-stranded DNA from nested deletions created from pIJ1933 and pIJ1934. DNA

Table 1. *R. leguminosarum* strains and plasmids

Strain or plasmid	Relevant characteristics	Reference/source
8401	<i>R. leguminosarum</i> lacking a symbiotic plasmid; Str ^R	Lamb <i>et al.</i> (1982)
A34	Derivative of 8401 carrying pRL1JI (previously called 8401/pRL1JI)	Downie <i>et al.</i> (1983)
A97	Derivative of A34 carrying <i>nodO94::Tn5-lacZ</i>	Economou (1990)
A131	Derivative of A34 carrying <i>nodC97::Tn5-phoA</i>	Economou (1990)
A146	Derivative of 8401 carrying a mutation that affects <i>nod</i> gene expression	This work
A150	Derivative of A146 containing pRL1JI carrying <i>nodC97::Tn5-phoA</i>	This work
A155	Derivative of A146 containing pRL1JI carrying <i>nodO94::Tn5-lacZ</i>	This work
A214	Derivative of A146 containing pJB5JI, (pRL1JI::Tn5)	This work
A273	Derivative of 8401 carrying <i>dctA21::Tn5-lacZ</i>	This work
A274	Derivative of 8401 carrying <i>dctB44::Tn5-lacZ</i>	This work
A275	Derivative of 8401 carrying <i>dctB5::Tn5-lacZ</i>	This work
A276	Derivative of 8401 carrying <i>dctB15::Tn5-lacZ</i>	This work
A281	Suc ⁻ derivative of A146 isolated after mutagenesis with pSUP102-Gm::Tn5-lacZ	This work
pIJ1089	cloned <i>nodABCDIJFELMNTO</i> genes from <i>R. l. viciae</i>	Downie <i>et al.</i> (1983)
pIJ1478	<i>nodD-lacZ</i> translational fusion	Rossen <i>et al.</i> (1985)
pIJ1687	Derivative of pIJ1089 carrying <i>nodC97::Tn5-phoA</i>	Economou (1990)
pIJ1848	Cosmid clone that complements the mutation in A146, carries <i>dctABD</i>	This work
pIJ1849	As pIJ1848, with which it overlaps	This work
pIJ1885	Deleted derivative of pIJ1848 contains a 10 kb <i>HindIII</i> fragment, carries <i>dctABD</i>	This work
pIJ1933	4.4 kb <i>AclI</i> from pIJ1885 subcloned in Bluescript SK(+)	This work
pIJ1934	As pIJ1933 but in the opposite orientation	This work
pIJ1968	Derivative of pIJ1848 carrying <i>dctB5::Tn5-lacZ</i>	This work
pIJ1969	Derivative of pIJ1848 carrying <i>dctB15::Tn5-lacZ</i>	This work
pIJ1970	Derivative of pIJ1848 carrying <i>dctA21::Tn5-lacZ</i>	This work
pIJ1971	Derivative of pIJ1848 carrying <i>dctB44::Tn5-lacZ</i>	This work
pIJ1991	Derivative of pIJ1848 containing the mutated region from A281	This work
pSUP102-Gm	Tet ^R Gm ^R , does not replicate in <i>R. leguminosarum</i>	Simon <i>et al.</i> (1989)
pSUP102-Gm::Tn5-lacZ	Derivative of pSUP102-Gm carrying Tn5-lacZ	Simon <i>et al.</i> (1989)

probes for hybridizations were labelled with ³²P by random priming of DNA fragments.

Nodulation tests. Measurements of nodulation and acetylene reduction by nodulated roots of peas were done as described by Johnston & Beringer (1975). Five-day-old nodules were prepared for light microscopy as described by Beringer *et al.* (1977).

RESULTS AND DISCUSSION

Isolation of a mutant with lowered *nod* gene expression

R. leguminosarum strain 8401, which lacks a symbiotic plasmid, was mutagenized with NTG. A plasmid (pIJ1687) carrying the entire *nod* gene region and the *nodC97::Tn5-phoA* allele, was transferred into the mutagenized population by conjugation; the transconjugants were then plated onto succinate minimal medium (Y) plates supplemented with streptomycin plus kanamycin

(to select transconjugants), hesperetin to induce the *nod* genes, and X-P to monitor the level of PhoA activity resulting from the expression of the NodC-PhoA fusion.

Of about 10 000 colonies screened, one that appeared pale blue compared with the background of dark blue colonies was confirmed to have a reduced level of PhoA activity by a quantitative assay in liquid medium. The reporter gene construct was then cured from the strain by screening for the loss of tetracycline resistance to yield mutant A146. When pIJ1687 was reintroduced into A146, the level of flavonoid-inducible PhoA was much less than that seen with colonies of the control (8401/pIJ1687), confirming that the mutation was in A146 rather than pIJ1687.

A derivative of pRL1JI containing the *nodC97::Tn5-phoA* allele was introduced into A146 by conjugation to form strain A150, and in parallel a different reporter system (β -galactosidase) was also introduced into A146

Table 2. Levels of *nod* gene induction in *R. leguminosarum*

The results shown are the means ($\pm$ SD) of five assays. –, Not applicable.

Strain	Alkaline phosphatase (units)		β -Galactosidase (units)	
	Hesperetin		Hesperetin	
	–	+	–	+
8401/pRL1JI, <i>nodC-phoA</i>	32 $\pm$ 3	660 $\pm$ 33	–	–
A146/pRL1JI, <i>nodC-phoA</i>	21 $\pm$ 1	169 $\pm$ 55	–	–
8401/pRL1JI, <i>nodO-lacZ</i>	–	–	76 $\pm$ 8	2305 $\pm$ 83
A146/pRL1JI, <i>nodO-lacZ</i>	–	–	65 $\pm$ 3	592 $\pm$ 76
8401/pIJ1478, <i>nodD-lacZ</i>	–	–	541 $\pm$ 39	–
A146/pIJ1478, <i>nodD-lacZ</i>	–	–	317 $\pm$ 12	–
8401/pRL1JI/pIJ1478, <i>nodD-lacZ</i>	–	–	390 $\pm$ 51	–
A146/pRL1JI/pIJ1478, <i>nodD-lacZ</i>	–	–	258 $\pm$ 19	–

using the *nodO94::Tn5-lacZ* allele on pRL1JI to form strain A155. As shown in Table 2, the levels of flavonoid-inducible β -galactosidase or PhoA in the mutant were about one-third of those of the isogenic control strains A131 (8401/pRL1JI, *nodC97::Tn5-phoA*) and A97 (8401/pRL1JI, *nodO94::Tn5-lacZ*). Since the reduction is seen with both reporter systems, the mutation must influence *nod* gene expression rather than, for example, processing of PhoA.

The level of *nodD* expression in A146 was measured by introducing the *nodD-lacZ* gene fusion on pIJ1478. This revealed that the level of *nodD* expression was about 60 % of normal (Table 2), indicating that the effect of the mutation was to reduce *nodD* expression rather than affecting NodD-mediated regulation (e.g. flavonoid uptake or processing). In *R. l. viciae*, NodD normally represses transcription from the *nodD* promoter (Rossen *et al.*, 1985); the introduction of *nodD* (on pRL1JI) into the mutant carrying pIJ1478 revealed that similar repression was maintained in the mutant (Table 2).

Effects of the mutation on symbiotic phenotype

pJB5JI is a derivative of pRL1JI carrying Tn5 in a bacteriocin gene, and it confers normal nodulation and nitrogen-fixation abilities on strains such as 8401 which lack a symbiotic plasmid (Johnston *et al.*, 1978). When strain A214, a derivative of A146 carrying pJB5JI, was inoculated onto pea seedlings, nodules appeared at the normal time but were smaller and more numerous than with the control strain (8401/pJB5JI). The nodules were pale pink or green (compared with the normal reddish-pink colour), and older plants had pale green leaves. These characteristics are typical of peas with abnormal nitrogen fixation. The nodules formed by the mutant were only 5–20 % as effective as the control in reducing acetylene.

Electron microscopy of thin sections of young nodules revealed that normal infection threads were present, but

Fig. 1. Electron micrographs of thin sections of pea nodules induced by (a) the control strain A34 and (b) the mutant A214 (A146 carrying pJB5JI). Bar, 4 μ m.

the number of bacteria within infected nodule tissue was much lower than normal; those bacteria present seemed to lack the normal plant-made peribacteroid membrane (Fig. 1). Therefore the mutation seemed to have blocked normal bacteroid development.

Fig. 2. Map of the region from pIJ1848 which complements A146. The 4.4 kb *AccI* region sequenced on pIJ1333 is indicated and the deduced positions of the open reading frames encoding *dctA*, *dctB* and *dctD* are shown. The open triangles indicate the positions of the *dctA*21, *dctB*44, *dctB*5 and *dctB*15 alleles mapped on pIJ1970, pIJ1971, pIJ1968 and pIJ1969, respectively. The filled triangle represents the mapped location of the element that caused the mutation in A146. The enlarged region illustrates the position (arrowheads) of the oligonucleotides used to map the insertion. The location of the deduced IS50 insertion element is indicated (heavy line), and the arrow shows the direction of transcription of the transposase (*tnp*) encoded on IS50R.

Characterization of the mutated region in A146

Strain A150 (the derivative of A146 carrying the *nodC97::Tn5-phoA* allele on pRL1JI) was used as a recipient in a conjugation with a population of *E. coli* containing random cosmid clones of *R. leguminosarum* DNA, and the transconjugants were screened on minimal medium containing hesperitin plus X-P to monitor induction of *nodC-phoA*. Two overlapping plasmids, pIJ1848 and pIJ1849, were identified which could restore normal flavonoid-inducible *nodC-phoA* expression to A150. When either of these plasmids was transferred to strain A155 (A146/pRL1JI, *nodO93::Tn5-lacZ*), normal levels of flavonoid-inducible β -galactosidase were restored. Both pIJ1848 and pIJ1849 also complemented the nitrogen-fixation defect of A146/pJB5JI. Therefore the region of DNA common to pIJ1848 and pIJ1849 complements both *nod* gene induction and nitrogen fixation, indicating that both phenotypes result from either a single mutation or closely linked mutations.

pIJ1848 was mutagenized with *Tn5-lacZ* and mutated derivatives were screened on plates for their ability to complement flavonoid-inducible *nodC-phoA* expression in strain A150. From about 700 independent mutant plasmids, four were identified (pIJ1968, pIJ1969, pIJ1970 and pIJ1971) that were unable to complement flavonoid-inducible *nodC-phoA* expression. DNA from each was digested with a variety of restriction enzymes; all four gave different patterns, each consistent with insertion of a single transposon. Using a variety of techniques, including digests with combinations of restriction enzymes, subcloning and DNA hybridizations, the sites of transposon insertions were mapped and found to be in a 4 kb region within a 10 kb *HindIII* fragment (Fig. 2) which was subcloned. Further mapping identified a 4.4 kb *AccI* fragment that spanned the region in which the four transposon-induced mutations were located (Fig. 2).

The 4.4 kb *AccI* fragment was subcloned in both orientations in Bluescript SK(+) to form pIJ1933 and

Table 3. Succinate uptake in *R. leguminosarum*

Strain	Succinate uptake [nmol min ⁻¹ (mg protein) ⁻¹] after growth on:	
	Glucose + NH ₄ ⁺	Glucose + aspartate
8401	7.1	43.0
A146	25.9	23.0
A277 (<i>dctA</i>)	0.5	4.7
A275 (<i>dctB</i>)	2.5	0.7
A281	2.4	0.0

pIJ1934. A set of nested deletions of each was made, and the DNA sequence was determined over most of the region. When this sequence and a translation of it were used to search for related sequences in the EMBL and GenBank databases, near identity (> 98%) was found with the *dct* gene region sequenced by Ronson *et al.* (1987a) from another strain of *R. l. viciae*, and high homology (approximately 80%) was found with the *dctA* and *dctB* genes from *R. meliloti*. The deduced positions of the *dctA*, *dctB* and *dctD* open reading frames are shown in Fig. 2. Three of the four mutations identified are in the *dctB* gene, and one is in *dctA*. These two genes are in different operons, *dctB* being one of two genes controlling *dctA* expression. The observation that derivatives of pIJ1848 mutated in *dctB* or *dctA* cannot complement the mutation in A146, indicates that this mutation may affect both *dctB* and *dctA*.

Measurements of succinate-dependent growth and succinate transport

The nature of the gene region identified was very surprising since the original mutant A146 had been isolated following a screen on Y medium containing succinate as C-source and glutamate as N-source; a *dct* mutant would not be expected to grow well on this medium (Ronson *et al.*, 1981). The growth of A146 in liquid minimal medium containing succinate and NH₄⁺ as the N-source was measured and found to be normal (data not shown).

The rates of succinate uptake were measured in cells grown in minimal medium with glucose plus NH₄⁺; surprisingly, the mutant had an uptake rate considerably higher than the control (Table 3). Under these growth conditions the *dct* transport system is not normally strongly induced (Watson *et al.*, 1993). Since aspartate is an efficient inducer of the *dct* genes (Watson *et al.*, 1993), the cells were grown on glucose plus aspartate (instead of NH₄⁺) as the N-source. This resulted in a strong induction of succinate transport in the control (8401), but had no effect in the mutant A146. Therefore A146 contains a mutation that causes constitutive expression of succinate uptake and does not reduce its ability to grow on succinate.

Fig. 3. Analysis of the DNA mutated in *R. leguminosarum* A146 and A281. Lanes A–E are autoradiographs of genomic DNA obtained following hybridization using ^{32}P -labelled pIJ1848 as a probe. Lanes: A, 8401 DNA cut with *Eco*RI; B, A146 DNA cut with *Eco*RI; C, 8401 DNA cut with *Bam*HI; D, A146 DNA cut with *Bam*HI; E, A281 DNA cut with *Bam*HI. Lanes F and G, an ethidium-bromide-stained gel of *Eco*RI-digested DNA from pIJ1848 and pIJ1991, respectively. The sizes of relevant fragments are indicated in kb.

Mutagenesis of A146 to isolate *Suc*[−] mutants and the phenotypes of other *dct* mutants

In view of the above results, we thought that there might be two succinate transport systems in the parental strain, one of which was being expressed constitutively in A146. This was addressed in two ways. The *dctA* allele from pIJ1970 and the *dctB* alleles from pIJ1968, pIJ1969 and pIJ1971 were transferred by homologous recombination into the chromosome of 8401. The resulting strains were tested for growth and uptake of succinate. A277 (carrying the *dctA* allele) did not grow on succinate and had no (or at most, very low) succinate uptake (Table 3). A275 (carrying one of the *dctB* alleles) grew very slowly on succinate/ NH_4^+ agar plates and had a very low level of succinate uptake (Table 3). Therefore mutations in *dctA* or *dctB* abolished (or severely lowered) succinate growth and uptake in these strains, indicating that there are not two succinate transport systems in 8401. An alternative strategy was to mutagenize A146 with Tn5-*lacZ* and screen for *Suc*[−] mutants. A surprisingly high frequency (1%) of *Suc*[−] mutants was found among the progeny of the mutagenesis by the suicide plasmid pSUP102Gm::Tn5-*lacZ*. One such mutant was called A281 and shown to have very low (if any) succinate uptake when grown with or without aspartate to induce the *dct* genes (Table 3). Like several other *Suc*[−] mutants isolated after mutagenesis of A146, A281 had a much higher frequency of reversion to *Suc*⁺ than expected or

Fig. 4. Identification of part of Tn5 in *R. leguminosarum* A146. Autoradiographs of hybridizations with ^{32}P -labelled pSUP102-Gm, (lanes A–C) and hybridizations with ^{32}P -labelled pSUP202-Gm::Tn5 (lanes D–F). *Eco*RI-digested DNA was from strain 8401 (lanes A and D), strain A146 (lanes B and E) and strain A281 (lanes C and F).

seen with other *Suc*[−] *dct* mutants such as A275 or A277. Strain A281 could be restored to normal growth by pIJ1848 carrying the *dct* region.

The high frequency of appearance of *Suc*[−] mutants following mutagenesis of A146, and their relatively high reversion rates, indicated that some rearrangements may have occurred at the *dct* locus. *Eco*RI- or *Bam*HI-digested DNA from A146 and the control 8401 was probed with pIJ1848. This revealed that the *Bam*HI fragment of about 14 kb in 8401 was increased in size (to about 16 kb) in A146 and that an *Eco*RI fragment of 4.8 kb was replaced by a fragment of 6.5 kb (Fig. 3). This 4.8 kb *Eco*RI fragment corresponds to that carrying part of *dctB* plus *dctD* (Fig. 2). Therefore the original mutation in A146 was probably caused by the insertion of an approximately 1.7 kb fragment. The presence of this insertion was unexpected since A146 had been mutagenized with NTG. However, the phenotype had been identified following the introduction of pIJ1687 carrying Tn5-*phoA*, a derivative of Tn5, in *nodC*. Therefore it was theoretically possible that the insertion might have arisen from insertion of part of the transposon on pIJ1687, and significantly the IS50R element from Tn5-*phoA* is 1.7 kb in size (Reznikoff, 1993). Hybridization with the *dct* region on pIJ1848 revealed that the mutation in A281 caused a further alteration, with the approximately 16 kb *Bam*HI fragment being replaced by a larger fragment (Fig. 3). These observations could be explained if Tn5-*lacZ* on the suicide plasmid had recombined with a region of homology (e.g. IS50) in the *dct* region in A146. Such a recombination-mediated mutation could explain the high frequency of appearance of *Suc*[−] mutants, and the resolution of such a cointegrate by recombination would result in a relatively high level of *Suc*⁺ 'revertants'.

*Eco*RI-digested DNA from 8401, A146 and A281 was probed with the vector pSUP102-Gm and a derivative of it carrying Tn5 (Fig. 4). As expected, no hybridization

was seen in either case with DNA from 8401. With A146, a hybridizing band was seen only when the derivative carrying Tn5 was used as a probe, confirming that part of Tn5 was present in A146. This 6.5 kb fragment is the same size as the novel fragment identified in A146 using a *dctB* gene probe. With the 'double' mutant A281, hybridization was seen with both pSUP102-Gm and pSUP102-Gm::Tn5 (Fig. 3). The different patterns seen with 8401, A146 and A281 are consistent with the hypothesis that the entire replicon (pSUP102-Gm including Tn5-*lacZ*) had integrated into A146 to form A281, and mutant A281 was indeed found to be resistant to gentamicin (presumably from pSUP102-Gm).

The mutated region from A281 was recombined into pIJ1848 by preparing DNA from A281/pIJ1848 and using it to transform *E. coli*, selecting for resistance to both tetracycline (on pIJ1848) and kanamycin (on Tn5-*lacZ*). The sizes of *EcoRI* fragments in plasmids from several transformants were found to be identical. The results with one representative clone (pIJ1991) are shown in Fig. 3. The 4.8 kb *EcoRI* fragment containing the *dctBD* gene region is replaced by three novel fragments of sizes 5.9 kb (seen as a doublet), 6.5 kb and 9.6 kb. This would be consistent with the integration of pSUP102-Gm::Tn5-*lacZ* into A146 to form A281, and the entire region being recombined onto pIJ1848 to form pIJ1991. Also compatible with this idea were the observations that pIJ1991 carries gentamicin resistance (from pSUP102-Gm), and that in *E. coli*, pIJ1991 appeared to have a higher copy number than pIJ1848, presumably as a result of the increased level of replication conferred by the pSUP102-Gm replicon.

We made the assumption that the initial mutation in A146 was caused by IS50 from the Tn5-*phoA*, and that the subsequent event in A281 was recombination via IS50 sequences in A146 and pSUP102-Gm::Tn5-*lacZ*. Restriction mapping indicated that the initial insertion must be at the left end of the 4.8 kb *EcoRI* fragment, within *dctB*. A PCR was done using one primer homologous to *dctB*, close to the *EcoRI* site in the 4.8 kb *EcoRI* fragment, and a second primer homologous to the end of IS50, priming outward (Fig. 2). With DNA from A146 and pIJ1991, a 0.6 kb fragment was found, whereas no products were found with either of the single primers. The insertion event is therefore most probably about 0.6 kb from the *EcoRI* site, about 1200 nucleotides downstream of the ATG that initiates the 1866-nucleotide sequence predicted to encode DctB.

Allele-specificity effect on *nod* gene induction

Other mutations have been described within *dctB*, but no effects on *nod* gene induction have been reported. The *nodD-lacZ* fusion on pIJ1478, and the *nodC-phoA* fusion on pIJ1687, were each transferred to the *dctB* mutant strain A275 and the *dctA* and *dctB* mutant derivatives (CR534 and CR535, respectively) from another strain (3855) of *R. l. viciae* (Ronson *et al.*, 1984). In each case, the levels of *nod* gene induction with the *dct* mutants were similar to those for the isogenic control strains.

The *R. leguminosarum* transducing phage RL38 was plated on the *dctB* mutant A275 and used to transduce A146 to kanamycin resistance. The levels of *nodD* expression and *nodC* gene induction in 20 transductants were measured after transferring pIJ1478 and pIJ1687 into them. In each case, *nod* gene expression levels were normal in the transductants. Replacement of the *dctB* allele in A146 with the *dctB5::Tn5-lacZ* allele from A275 was accompanied by restoration of normal *nod* gene induction, thus confirming linkage between the mutation in the *dctB* region of A146 and the lowered *nod* gene induction. This also reconfirmed that the 'normal' *dctB* mutation in A275 does not influence either *nodD* expression or *nodC* induction. Therefore the effect of the mutation in A146 on *nod* gene induction is allele-specific.

It is not immediately obvious why a mutation in *dctB* should have an effect on *nod* gene expression. DctB along with DctD is required for the succinate-dependent induction of *dctA* (Ronson *et al.*, 1987b; Yarosh *et al.*, 1989; Jiang *et al.*, 1989). *dctB* is also required for aspartate-dependent induction of succinate transport (Watson *et al.*, 1993). The mutation in A146 appears to cause constitutive succinate uptake. However, although this mutation allows succinate uptake (at a level about 50% of that seen after maximal induction with aspartate), the ability of the mutant to fix nitrogen in nodules is severely impaired.

It would appear that the regulation of the *dctA* gene is complex, because in addition to regulatory effects of the two-component regulatory system encoded by *dctB* and *dctD*, mutation of *dctA* itself causes high-level expression from the *dctA* promoter, even in cells not exposed to dicarboxylic acids (Ronson & Astwood, 1985; Yarosh *et al.*, 1989; Jiang *et al.*, 1989). The high level of succinate transport in A146 grown on medium containing glucose plus NH₄⁺ indicates that the *dctA* gene is probably expressed at a high level. In this regard the mutation is analogous to the effect of a *dctA* mutation, but evidently in A146 the *dctA* gene product is functional since high levels of succinate transport are observed. In fact it is rather surprising that although the mutation appears to cause constitutive succinate transport, nitrogen fixation by mutant bacteroids is severely impaired. Thus, although succinate uptake is necessary for nitrogen fixation, it appears that higher-level, or properly regulated, expression is required.

The mutation of *dctB* in A146 appears to be allele-specific since other mutations in *dctB* do not have a similar effect on *nod* gene expression. Such allele specificity could be explained at the DNA level, e.g. by the creation of a promoter that causes constitutive *dctA* expression, and such an effect would be consistent with the complementation observations which indicate that the mutation in *dctB* has a *cis* effect on *dctA*. To address the possibility that constitutive expression of *dctA* reduces the expression of *nodD* and other *nod* genes, plasmid pRU296 (which carries *dctA* expressed constitutively from the neomycin phosphotransferase promoter in the vector pML122) was transferred to strains 8401/pIJ1478 (*nodD-lacZ*) and A97 (*nodO-lacZ*). The levels of *nod* gene expression were measured as described in Table 2 and found to be normal;

strain 8401/pIJ1478/pRU296 expressed 524 (± 42) units of β -galactosidase activity, and strain A97/pRU296 expressed 2286 (± 92) units of β -galactosidase after growth in the presence of hesperetin. Therefore it is unlikely that the reduction in *nod* gene expression in A146 is simply due to constitutive *dctA* expression.

An alternative explanation for the allele-specific nature of the *dctB* mutation in A146 is that a truncated DctB protein (or proteins) might be made. The estimated position of the putative IS50 mutation in A146 is such that it could result in the formation of a derivative of DctB which lacks the domain that normally interacts with DctD, but could retain the two membrane-spanning regions that locate a large loop of DctB in the periplasm. It is also possible that only the C-terminal part of DctB could be formed, and such a deleted protein might retain auto-phosphorylation activity and phosphorylate DctD, resulting in constitutive *dctA* expression. Such a mutant DctB protein might even phosphorylate other analogous regulatory proteins. Sanjuan *et al.* (1994) have demonstrated that the *nodD1* gene in *Bradyrhizobium japonicum* is under the control of the *nodV* and *nodW* genes, which encode a two-component regulatory system analogous to that encoded by *dctB* and *dctD*. If similar regulation of *nodD* were present in *R. leguminosarum*, it is possible that a truncated form of DctB might alter the phosphorylation state of a transcriptional activator such as NodW, and this could result in lowered *nodD* expression. Such a model is consistent with the allele-specific nature of the *dctB* mutation in A146. However it does not necessarily explain why the mutation in A146 should have a *cis* effect on *dctA*. This might be explained if there were normally some interaction between DctA and DctB, and an abnormal interaction occurred between DctA and a deleted form of DctB.

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