**Rhizobium leguminosarum** nodulation gene (*nod*) expression is lowered by an allelespecific 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 (pPJ1848) from a *R. leguminosarum* cosmid library. DNA hybridization confirmed that pPJ1848 was not from the symbiotic plasmid and showed that a DNA insertion was present in the mutant. The complementing region of pPJ1848 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 allelespecific 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

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**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 birsuta* (Gottfert, 1993). In one well-characterized strain of *R. l. viciae*, 13 nod genes have been identified (Downie, 1991). Twelve of these, in four operons (*nod*ABCJ, *nod*FEL, *nod*MNT and *nod*O), 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; Spanik et al., 1987). Circumstantial evidence indicates that there is a direct interaction between the flavonoids and NodD (Burn et al., 1987; Spanik 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, NH4+ 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 result 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 (Beringer, 1974), or Y minimal medium supplemented with 10 mM sodium glutamate, sodium aspartate or NH4Cl 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 μg ml−1; kanamycin, 20 μg ml−1; tetracycline, 5 μg ml−1; gentamicin, 5 μg ml−1; carbencillin, 10 μg 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 hesperitin and 40 μg 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 NH4Cl 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 x 10⁸ cells ml−1) in Y medium containing glucose and NH4Cl 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 [2,3-14C]sucinate (40 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 & Helsinki, 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 hesperitin 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 *det* genes by homologous recombination as described by Ruvkun & Ausubel (1981); recombinants were checked for the appropriate pattern of 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 NH4Cl. The mutations in the *det* 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 HindIII and re-ligating the DNA and screening for DNA fragments present. Only a 1 kb HindIII fragment was released by HindIII digestion of pIJ1885. This plasmid was digested with EcoRI, and a 4.4 kb fragment was subcloned in either orientation into phagemid Bluescript SK (+) (Strategene) to form pIJ1933 and pIJ1934.

PCR amplification of DNA from pIJ1991 and A146 was done using the oligonucleotide 5'-GGAGGTCACTGAGGAGT-CAG, which is homologous to nucleotides 46–65 from the end of Tn5, and the oligonucleotide 5'-CGGCACCCCGTCTCAGTTGCG, which is homologous to nucleotides 759–778 of the *detB* region as described by Ronson et al. (1987). Following an initial denaturation for 5 min at 95 °C, conditions were 34 cycles of 45 s at 95 °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, 5 mM MgCl₂, 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

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td>8401</td>
<td><em>R. leguminosarum</em> lacking a symbiotic plasmid; Str&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Lamb et al. (1982)</td>
</tr>
<tr>
<td>A34</td>
<td>Derivative of 8401 carrying pRL1JI (previously called 8401/pRL1JI)</td>
<td>Downie et al. (1983)</td>
</tr>
<tr>
<td>A97</td>
<td>Derivative of A34 carrying nodO94::Tn5-lacZ</td>
<td>Economou (1990)</td>
</tr>
<tr>
<td>A131</td>
<td>Derivative of A34 carrying nodC97::Tn5-phoA</td>
<td>Economou (1990)</td>
</tr>
<tr>
<td>A146</td>
<td>Derivative of 8401 carrying a mutation that affects nod gene expression</td>
<td>This work</td>
</tr>
<tr>
<td>A150</td>
<td>Derivative of A146 containing pRL1JI carrying nodC97::Tn5-phoA</td>
<td>This work</td>
</tr>
<tr>
<td>A155</td>
<td>Derivative of A146 containing pRL1JI carrying nodO94::Tn5-lacZ</td>
<td>This work</td>
</tr>
<tr>
<td>A214</td>
<td>Derivative of A146 containing p[JB5Jl (pRL1JI::Tn5)</td>
<td>This work</td>
</tr>
<tr>
<td>A273</td>
<td>Derivative of 8401 carrying dctA21::Tn5-lacZ</td>
<td>This work</td>
</tr>
<tr>
<td>A274</td>
<td>Derivative of 8401 carrying dctB44::Tn5-lacZ</td>
<td>This work</td>
</tr>
<tr>
<td>A275</td>
<td>Derivative of 8401 carrying dctB5::Tn5-lacZ</td>
<td>This work</td>
</tr>
<tr>
<td>A276</td>
<td>Derivative of 8401 carrying dctB15::Tn5-lacZ</td>
<td>This work</td>
</tr>
<tr>
<td>A281</td>
<td>Suc&lt;sup&gt;−&lt;/sup&gt; derivative of A146 isolated after mutagenesis with pSUP102-Gm::Tn5-lacZ</td>
<td>This work</td>
</tr>
<tr>
<td>pIJ1089</td>
<td>Cloned nodABCDIJFEJLMNTO genes from <em>R. l. viciae</em></td>
<td>Downie et al. (1983)</td>
</tr>
<tr>
<td>pIJ1478</td>
<td>nodD-lacZ translational fusion</td>
<td>Rossen et al. (1985)</td>
</tr>
<tr>
<td>pIJ1687</td>
<td>Derivative of pIJ1089 carrying nodC97::Tn5-phoA</td>
<td>Economou (1990)</td>
</tr>
<tr>
<td>pIJ1848</td>
<td>Cosmid clone that complements the mutation in A146, carries dctABD</td>
<td>This work</td>
</tr>
<tr>
<td>pIJ1849</td>
<td>As pIJ1848, with which it overlaps</td>
<td>This work</td>
</tr>
<tr>
<td>pIJ1885</td>
<td>Deleted derivative of pIJ1848 contains a 10 kb HindIII fragment, carries dctABD</td>
<td>This work</td>
</tr>
<tr>
<td>pIJ1933</td>
<td>4.4 kb AceI from pIJ1885 subcloned in Bluescript SK(+)</td>
<td>This work</td>
</tr>
<tr>
<td>pIJ1934</td>
<td>As pIJ1933 but in the opposite orientation</td>
<td>This work</td>
</tr>
<tr>
<td>pIJ1968</td>
<td>Derivative of pIJ1848 carrying dctB5::Tn5-lacZ</td>
<td>This work</td>
</tr>
<tr>
<td>pIJ1969</td>
<td>Derivative of pIJ1848 carrying dctB15::Tn5-lacZ</td>
<td>This work</td>
</tr>
<tr>
<td>pIJ1970</td>
<td>Derivative of pIJ1848 carrying dctA21::Tn5-lacZ</td>
<td>This work</td>
</tr>
<tr>
<td>pIJ1971</td>
<td>Derivative of pIJ1848 carrying dctB44::Tn5-lacZ</td>
<td>This work</td>
</tr>
<tr>
<td>pIJ1991</td>
<td>Derivative of pIJ1848 containing the mutated region from A281</td>
<td>This work</td>
</tr>
<tr>
<td>pSUP102-Gm</td>
<td>Tet&lt;sup&gt;R&lt;/sup&gt; Gm&lt;sup&gt;R&lt;/sup&gt;, does not replicate in <em>R. leguminosarum</em></td>
<td>Simon et al. (1989)</td>
</tr>
<tr>
<td>pSUP102-Gm::Tn5-lacZ</td>
<td>Derivative of pSUP102-Gm carrying Tn5-lacZ</td>
<td>Simon et al. (1989)</td>
</tr>
</tbody>
</table>

probes for hybridizations were labelled with <sup>32</sup>P 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 10000 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 (±sd) of five assays. –, Not applicable.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Alkaline phosphatase (units)</th>
<th>β-Galactosidase (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hesperetin</td>
<td>Hesperetin</td>
</tr>
<tr>
<td>8401/pRL1JI, nodC-phoA</td>
<td>32 ± 3</td>
<td>660 ± 33</td>
</tr>
<tr>
<td>A146/pRL1JI, nodC-phoA</td>
<td>21 ± 1</td>
<td>169 ± 55</td>
</tr>
<tr>
<td>8401/pRL1JI, nodO-lacZ</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>A146/pRL1JI, nodO-lacZ</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>8401/pIJ1478, nodD-lacZ</td>
<td>–</td>
<td>76 ± 8</td>
</tr>
<tr>
<td>A146/pIJ1478, nodD-lacZ</td>
<td>–</td>
<td>65 ± 3</td>
</tr>
<tr>
<td>8401/pRL1JI/pIJ1478, nodD-lacZ</td>
<td>–</td>
<td>390 ± 51</td>
</tr>
<tr>
<td>A146/pRL1JI/pIJ1478, nodD-lacZ</td>
<td>–</td>
<td>258 ± 19</td>
</tr>
</tbody>
</table>

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, nodC77::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 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.
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 conjugal transfer with a population of E. coli containing random cosmids 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/pIJ153JI. 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 100 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 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 det gene region sequenced by Ronson et al. (1987a) from another strain of R. leguminosarum, and high homology (approximately 80%) was found with the detA and detB genes from R. meliloti. The deduced positions of the detA, detB and detD open reading frames are shown in Fig. 2. Three of the four mutations identified are in the detB gene, and one is in detA. These two genes are in different operons, detB being one of two genes controlling detA expression. The observation that derivatives of pIJ1848 mutated in detB or detA cannot complement the mutation in A146, indicates that this mutation may affect both detB and detA.

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 screening on Y medium containing succinate as C-source and glutamate as N-source; a det 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⁺ 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.
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/\text{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 uptake (Table 3).

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 32P-labelled pIJ1848 as a probe. Lanes: A, 8401 DNA cut with EcoRI; B, A146 DNA cut with EcoRI; C, 8401 DNA cut with BamHI; D, A146 DNA cut with BamHI; E, A281 DNA cut with BamHI. Lanes F and G, an ethidium-bromide-stained gel of EcoRI-digested DNA from pIJ1848 and pIJ1991, respectively. The sizes of relevant fragments are indicated in kb.

Therefore mutations in dctA or dctB abolished (or severely lowered) succinate 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 pSUP102-Gm::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 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. EcoRI- or BamHI-digested DNA from A146 and the control 8401 was probed with pIJ1848. This revealed that the BamHI fragment of about 14 kb in 8401 was increased in size (to about 16 kb) in A146 and that an EcoRI fragment of 4.8 kb was replaced by a fragment of 6.5 kb (Fig. 3). This 4.8 kb EcoRI 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 BamHI 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’.
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 A282 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. leguminosarum (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 dctB::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/pJ1478/pRU296 expressed 524 (+42) units of $\beta$-galactosidase activity, and strain A97/pRU296 expressed 2286 (+92) units of $\beta$-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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