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Cloning of the symbiotic region of *Rhizobium leguminosarum*: the nodulation genes are between the nitrogenase genes and a *nifA*-like gene

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The region of the *Rhizobium leguminosarum* plasmid pRL1JI involved in nodulation and nitrogen fixation has been cloned on a series of four overlapping cosmid clones. These clones represent ~60 kb of pRL1JI DNA on which a series of Tn5-induced *fix* and *nod* alleles have been identified, with the two most distant alleles being separated by ~45 kb of DNA. The mutant alleles fell into three groups, two clusters of *fix* alleles separated by one cluster of *nod* alleles. Within one group of *fix* alleles, DNA homologous to the *nifA* gene of *Klebsiella pneumoniae* has been identified, whereas the pRL1JI DNA homologous to the *K. pneumoniae* nitrogenase genes is present within the other group of *fix* alleles.
Key words: Rhizobium/nitrogen fixation/nodulation/*nifA* gene

Introduction

The genes involved in nitrogen fixation and nodulation of leguminous plants by the fast-growing species of *Rhizobium* appear to be closely linked on large plasmids in those species studied. A deletion of ~50 kb in one such plasmid in *R. leguminosarum* led to the loss of both nodulation and nitrogen fixation genes (Hirsch *et al.*, 1980; Buchanan-Wollaston *et al.*, 1980). Similar observations were made using strains of *R.meliloti* containing deletions in their symbiotic plasmids (Banfalvi *et al.*, 1981; Rosenberg *et al.*, 1981). Analysis of overlapping cosmid clones derived from *R. meliloti* showed that mutations affecting nodulation genes were ~20 kb from the nitrogenase structural genes (Long *et al.*, 1982).

The *R. leguminosarum* plasmid, pRL1JI, is highly transmissible and can complement various non-nodulating or non-fixing mutants of *R. leguminosarum* including mutants deleted for both nodulation and nitrogen fixation genes (Johnston *et al.*, 1978; Brewin *et al.*, 1980). When pRL1JI was transferred to *R. phaseoli* or *R. trifolii* (which normally nodulate *Phaseolus* beans or clover, respectively) these *Rhizobium* strains acquired the ability to form nitrogen-fixing nodules on peas, the normal host of *R. leguminosarum* (Johnston *et al.*, 1978). Several mutations affecting the nitrogen-fixation genes on pRL1JI have been described (Ma *et al.*, 1982). These mutants were isolated following the insertion of the transposon Tn5 and the sites of insertion of Tn5 were mapped by cloning the restriction endonuclease fragments containing the Tn5 insertions. Thus, two unlinked regions of plasmid pRL1JI were identified which were involved in nitrogen fixation. In this paper the wild-type genes corresponding to the nitrogen fixation and nodulation genes have been cloned on a series of overlapping cosmids which span ~60 kb of pRL1JI DNA. Analysis of the cloned DNA has established the relative positions of nitrogen fixation and nodulation genes and these cosmids were shown to correct various *fix* and *nod* mutants. In addition, one of the cosmids was shown to carry a region of DNA homologous to *nifA*, the gene which activates the *Klebsiella pneumoniae nif* genes.

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Results

Isolation of cosmid clones containing fix and nod genes

Previously, a series of 200 independent Tn5 insertions into plasmid pRL1JI was isolated and the phenotypes of the insertions were tested on plants (Ma *et al.*, 1982). From this experiment, nine *fix* mutants and three *nod* mutants were isolated (Table I) and found to be clustered as one group of *nod* alleles and two groups of *fix* alleles.

To determine the physical linkage of these groups of alleles, cosmid clones were constructed using DNA isolated from a strain of *R. leguminosarum* (B155) which contains pRL1JI. The clone bank was constructed using the cosmid vector pLAFRI which can replicate in *Rhizobium* species and *Escherichia coli* (Friedman *et al.*, 1982). The *Rhizobium* DNA was partially digested with *Eco*RI, fractionated into fragments of average size ~25 kb and ligated to the *Eco*RI-digested vector pLAFRI. The ligation mixture was packaged into phage λ as described by Hohn (1979) and ~2000 tetracycline-resistant transductants of *E. coli* strain ED8767 (Murray *et al.*, 1977) were isolated.

Table I. Bacterial strains and plasmids

Bacterial strains	Relevant characters	Source
R. leguminosarum		
6015	*phe trp rif str nod 6007* (deletion of *nod* and *fix* genes)	Johnston *et al.*, 1978
248	Field isolate containing pRL1JI	
3688	6015 (pRL1JI)	Isolated by mating 248 with 6015
IMA3	6015 (pRL1JI; *fix-3*::Tn5)	Independent Tn5 insertions into plasmid pRL1JI (Ma *et al.*, 1982; Downie *et al.*, 1983)
IMA15	6015 (pRL1JI; *fix-15*::Tn5)	
IMA16	6015 (pRL1JI; *fix-16*::Tn5)	
IMA17	6015 (pRL1JI; *fix-17*::Tn5)	
IMA37	6015 (pRL1JI; *fix-37*::Tn5)	
IMA52	6015 (pRL1JI; *fix-52*::Tn5)	
IMA57	6015 (pRL1JI; *nod-57*::Tn5)	
IMA67	6015 (pRL1JI; *fix-67*::Tn5)	
IMA128	6015 (pRL1JI; *nod-128*::Tn5)	
IMA137	6015 (pRL1JI; *fix-137*::Tn5)	
IMA183	6015 (pRL1JI; *nod-183*::Tn5)	
B151	Strain 128C53 cured of its symbiotic plasmid	Brewin *et al.*, 1982
B155	B151 (pRL1JI)	Brewin *et al.*, 1982
Plasmids		
pIJ1021	*Bam*HI fragment of pRL1JI adjacent to the *fix-3*::Tn5 insertion cloned in pACYC184	Ma *et al.*, 1982
pIJ1054	*Eco*RI fragment of pRL1JI carrying the *nod-128*::Tn5 allele cloned in pUR2	Downie *et al.*, 1983

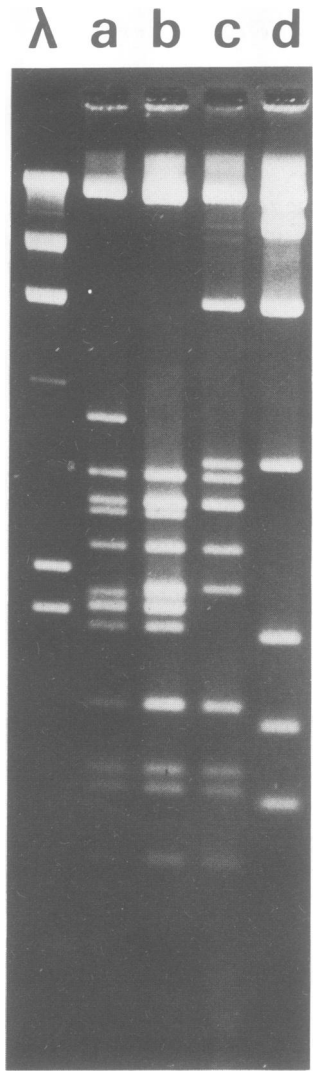

Fig. 1. *Eco*RI fragments present in plasmids carrying subcloned regions of pRL1JI. Plasmids pIJ1088 **(a)**, pIJ1086 **(b)**, pIJ1089 **(c)** and pIJ1085 **(d)** were digested with *Eco*RI and electrophoresed in a 1% agarose gel. The size standard was phage λ DNA digested with *Hind*III.

Colonies from the clone bank carrying nodulation or nitrogen fixation genes were identified by colony hybridisation (Grunstein and Hogness, 1975) using two different DNA probes. These probes were isolated from two recombinant plasmids: pIJ1021 which contains DNA adjacent to the *fix-3*::Tn5 allele (Ma *et al.*, 1982 and see Figure 2), and plasmid pIJ1054 which contains DNA flanking the *nod-128*::Tn5 allele (Downie *et al.*, 1983 and see Figure 2). When the gene bank was probed with the 1.1-kb *Hind*III fragment of *Rhizobium* DNA from plasmid pIJ1021, two colonies were identified containing recombinant plasmids which were designated pIJ1086 and pIJ1088. These plasmids were confirmed by DNA hybridisation to contain DNA which had previously been shown (Ma *et al.*, 1982) to be adjacent to the *fix-3*::Tn5 allele. Using the 2.2-kb *Bam*HI fragment from plasmid pIJ1054 (Figure 2) as a probe, two other plasmids designated pIJ1085 and pIJ1089 were identified, and these were confirmed by DNA hybridisation to contain the DNA previously shown to be adjacent to the *nod-128*::Tn5 allele.

When plasmids pIJ1085, pIJ1086, pIJ1088 and pIJ1089 were digested with *Eco*RI (Figure 1) many fragments of similar sizes were present in these plasmids. The estimated sizes of the fragments are listed in Table II. It appeared from these data that plasmids pIJ1086, pIJ1088 and pIJ1089 contained common DNA, and that plasmid pIJ1085 contained DNA in common with pIJ1089 but not with pIJ1086 or pIJ1088. It was confirmed that the same region of DNA had been cloned in these different plasmids using plasmids pIJ1086 and pIJ1089 as nick-translated probes to *Eco*RI digests of the four plasmids. Thus, it appears that the four plasmids comprise a group of overlapping clones representing pRL1JI DNA around the sites of Tn5-induced *fix* and *nod* mutant alleles. These four plasmids were confirmed by DNA hybridisation to contain only pRL1JI DNA since they hybridised with *Eco*RI-digested DNA from *R. leguminosarum* strain B155 (which contains pRL1JI) but not with DNA from *R. leguminosarum* strains B151 (which has lost its symbiotic plasmid) nor 6015 (which is deleted for the *nod* and *fix* genes).

Table II. Sizes of *Eco*RI fragments present in pIJ1085, pIJ1086, pIJ1088 and pIJ1089

pIJ1088	pIJ1086	pIJ1089	pIJ1085
			11.5
		6.6	6.6
3.7			
		3.20	3.20
3.15	3.15	3.15	
2.85	2.85	2.85	
	2.85	2.85	
2.75	2.75		
2.45	2.45	2.45	
	2.15	2.15	
2.10	2.10		
2.00	2.00		
2.00	2.00		
1.85	1.85		
			1.80
1.45	1.45	1.45	
	1.45	1.45	
			1.35
1.15	1.15	1.15	
1.05	1.05	1.05	
			1.00
0.80	0.80	0.80	
0.50	0.50		

The fragment sizes (in kilobase pairs) were estimated using phage λ cut with *Hind*III as a standard. The vector pLAFRI band (of 23 kb) is not listed.

Identification of the relative locations of the nod and fix genes

The restriction endonuclease map of the region of pRL1JI represented by these clones was established for the restriction enzymes *Eco*RI, *Hind*III and *Bam*HI. This map is shown in Figure 2 and was constructed using data from a series of sources: (a) by comparisons of single and double digests of pIJ1085, pIJ1086, pIJ1088 and pIJ1089 using *Eco*RI, *Bam*HI and *Hind*III; (b) using the restriction maps which had previously been established (Ma *et al.*, 1982; Downie *et al.*, 1983) of the regions of DNA around the sites of the Tn5 insertions; (c) by subcloning the *Hind*III fragments of plasmid pIJ1089 and mapping them with *Eco*RI and *Bam*HI and (d) by DNA hybridisation using these subclones of plasmid

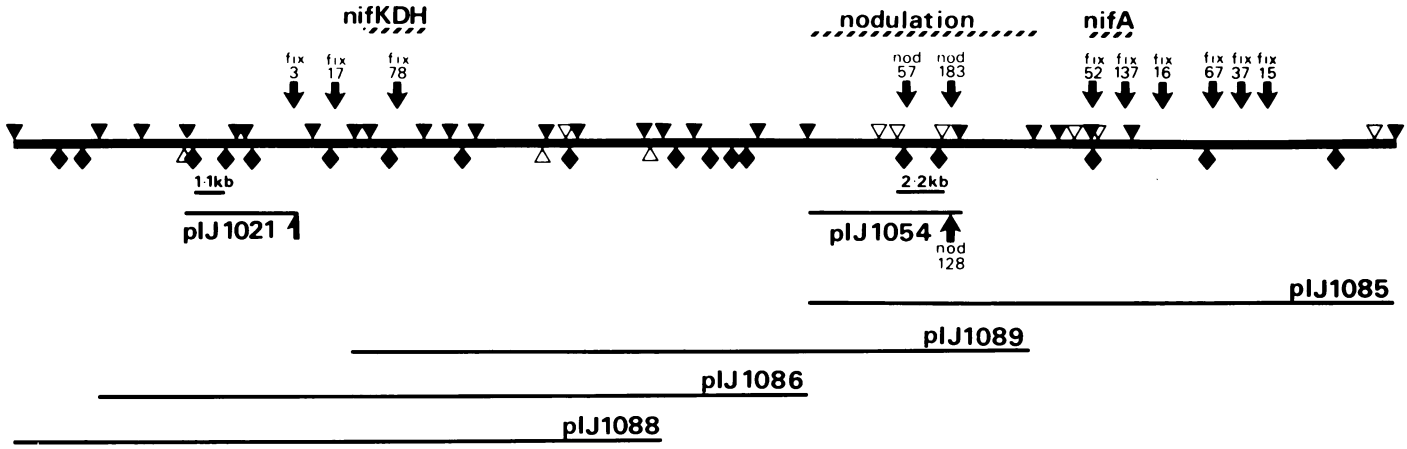

Fig. 2. Map of the region of pRL1JI DNA involved in nodulation and nitrogen fixation. The restriction endonuclease sites marked are *Eco*RI (▼), *Hind*III (◆), and *Bam*HI (▽). The sites of Tn5 insertions are indicated by arrows and the *nod* and *fix* alleles are shown; for convenience the site of the *nod-128*::Tn5 allele is shown on pIJ1054 rather than on pRL1JI. Plasmids pIJ1021 and pIJ1054 were isolated previously (Ma *et al.*, 1982; Downie *et al.*, 1983), and fragments from them (1.1-kb *Hind*III fragment and 2.2-kb *Bam*HI fragment as shown) were used to identify the four cosmid clones pIJ1088, pIJ1086, pIJ1089 and pIJ1085. The regions of homology to the *nifA* gene and *nifKDH* genes of *K. pneumoniae* are indicated by broken lines and the region involved in nodulation (Downie *et al.*, 1983) is also shown.

pIJ1089 as probes.

In previous studies, 12 Tn5-induced symbiotically-defective mutants had been located in three groups: *fix-3*::Tn5, *fix-17*::Tn5 and *fix-78*::Tn5 in one *Bam*HI fragment; *fix-52*::Tn5, *fix-137*::Tn5, *fix-16*::Tn5, *fix-67*::Tn5, *fix-37*::Tn5 and *fix-15*::Tn5 in two adjacent *Eco*RI fragments, and *nod-57*::Tn5, *nod-128*::Tn5 and *nod-183*::Tn5 in one *Eco*RI fragment (Ma *et al.*, 1982; Downie *et al.*, 1983). By DNA hybridisation it was confirmed that the *Eco*RI fragments containing these alleles were present on one or more of the overlapping recombinant plasmids. The positions of the mutant alleles on the restriction map (Figure 2) were thus identified.

Identification of DNA homologous to nifA of K. pneumoniae

The *nifA* and *nifL* genes from *K. pneumoniae* are involved in the regulation of the nitrogen-fixation genes (Buchanan-Wollaston *et al.*, 1981) and the *nifA* gene and part of the *nifL* gene have been subcloned on plasmid pMC71A (Buchanan-Wollaston *et al.*, 1981). The *nifA* gene is within a 1.6-kb *Sma*I-*Kpn*I fragment and part of the *nifL* gene is present on a 1.0-kb *Kpn*I-*Sal*I fragment (F.C. and M.C.Cannon, personal communication). Each of these two fragments was purified by gel electrophoresis, nick-translated and used as a hybridisation probe. Using the *nifA* probe, one region of plasmid pIJ1085 was found to hybridise and was identified as the 4.8-kb *Hind*III fragment or the 1.8-kb *Eco*RI fragment (Figure 3). No homology was observed when the *nifL* probe was used. The 1.8-kb *Eco*RI fragment of plasmid pIJ1085 was subcloned, nick-translated and used to probe digests of plasmid pMC71A. The 1.6-kb *Sma*I-*Kpn*I *(nifA)* fragment was found to hybridise but the 1.0-kb *Kpn*I-*Sal*I fragment did not (not shown).

The nitrogenase genes from *K. pneumoniae* were previously found to hybridise to the DNA adjacent to the *fix-78*::Tn5 allele (Ma *et al.*, 1982) and this region of homoogy is also shown on the map (Figure 2).

Electron microscopy of nodules

Previously, we reported that in nodules induced by mutant

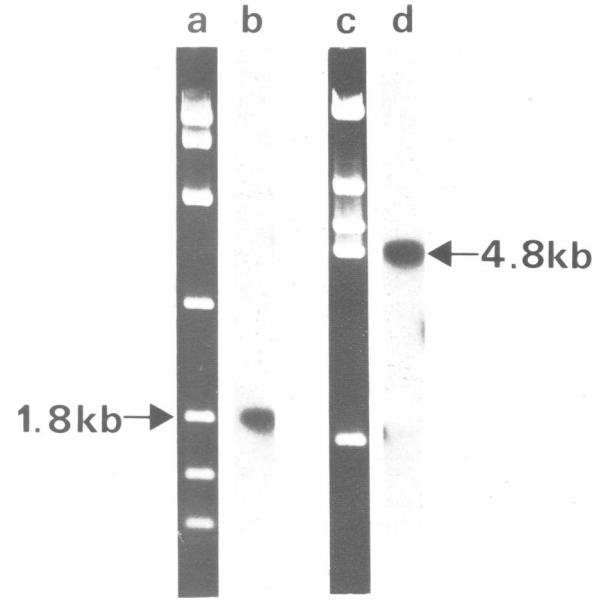

Fig. 3. Homology of the *K. pneumoniae nifA* gene to pIJ1085. Plasmid pIJ1085 was digested with *Eco*RI **(a)** or *Hind*III **(c)**, transferred to nitrocellulose and probed using the *Sma*I-*Kpn*I fragment of pMC71A containing the *nifA* gene. The hybridisation was carried out for 48 h at 57°C in 0.45 M NaCl, 0.045 M Na citrate pH 7.2 (3 x SSC). **Lanes (b)** and **(d)** show the *Eco*RI and *Hind*III fragments which hybridised.

strains carrying the alleles *fix-137*::Tn5, *fix-16*::Tn5, *fix-67*::Tn5, *fix-37*::Tn5 and *fix-15*::Tn5 the bacteria did not appear to be surrounded by a peribacteroid membrane (Ma *et al.*, 1982). However, a more detailed study showed that the nodules induced by these mutant strains did contain bacteria surrounded by a peribacteroid membrane (Figure 4a). Early in nodule development (Figure 4a), bacteria surrounded by a membrane could be seen, but this membrane was degraded after a few days (Figure 4c). Even in relatively old (4 weeks) nodules induced by wild-type strains the bacteroids remained surrounded by a membrane. The mutant affected in the nitrogenase gene region (*fix-78*::Tn5) appeared to have an intermediate phenotype, in that the peribacteroid membrane remain-

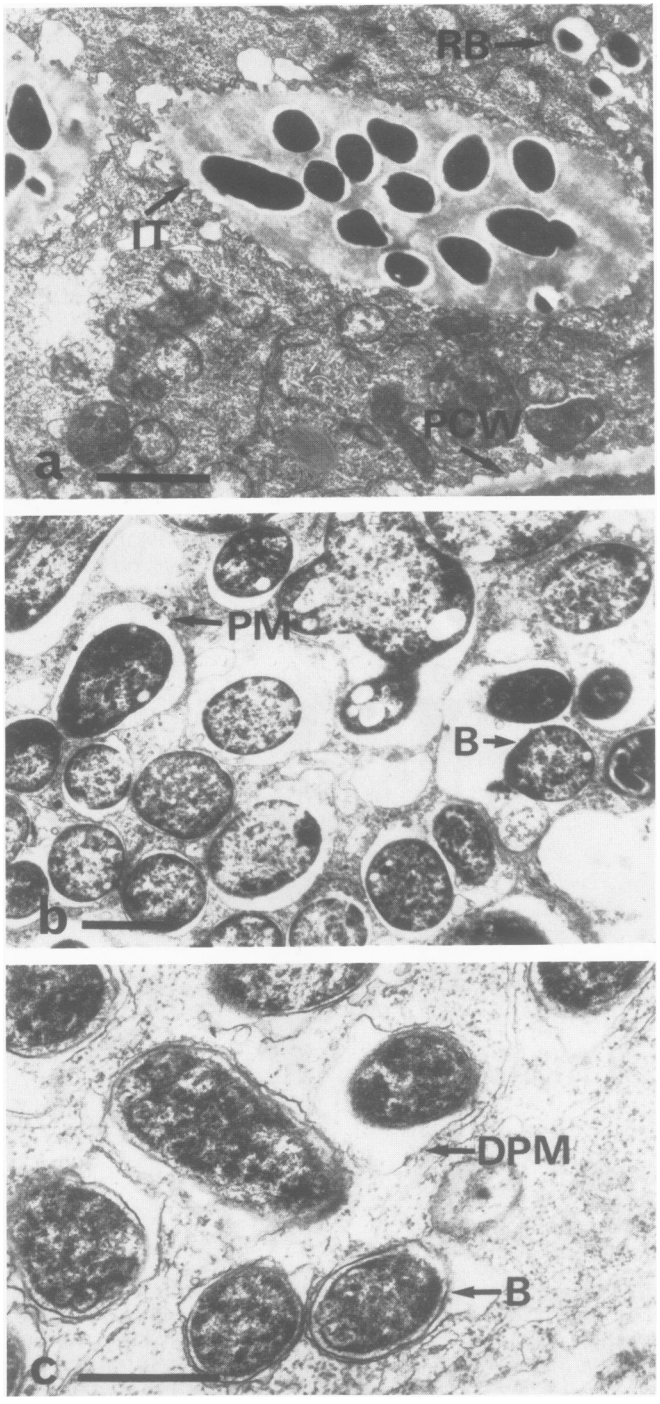

Fig. 4. Electron microscopy of nodules. Thin sections of nodules from peas were taken 3 days **(a)**, 15 days **(b)**, or 6 days **(c)**, after development of nodules. **(a)** and **(c)**, nodules induced by strain IMA67 (*fix-67*::Tn5). **(b)**, nodules induced by strain IMA78 (*fix-78*::Tn5). Abbreviations: IT, infection thread, RB, released bacteria; PCW, plant cell wall; PM, peribacteroid membrane; B, bacteroid; DPM, disintegrating peribacteroid membrane. The bars in **(a)** and **(b)** represent 2 μm and in **(c)** 5 μm.

ed intact for ~10 days after formation of the nodule (Figure 4b).

Nitrogen fixation in nodules induced by fix mutant strains carrying pIJ1085 or pIJ1086

Plasmid pIJ1085 was transferred to the mutant strains carrying the *fix-52*::Tn5, *fix-137*::Tn5, *fix-16*::Tn5, *fix-67*::Tn5, *fix-37*::Tn5 or *fix-15*::Tn5 alleles and plasmid pIJ1086 was transferred to strains carrying the *fix-3*::Tn5, *fix-17*::Tn5 or *fix-78*::Tn5 alleles. The transconjugants were inoculated onto plants and in each case the levels of nitrogen fixation were estimated using the acetylene reduction assay (Table III).

Table III. Levels of acetylene reduction and percentage of tetracycline-resistant bacteria in nodules induced by mutant strains carrying pIJ1085 or pIJ1086

Strain (allele)	Plasmid	Acetylene reduction (%)	Tetracycline-resistance (%)
3688 (W.T.)	–	100	n.t.
IMA3 (*fix-3*::Tn5)	pIJ1086	71	90
IMA17 (*fix-17*::Tn5)	pIJ1086	97	88
IMA78 (*fix-78*::Tn5)	pIJ1086	55	87
IMA52 (*fix-52*::Tn5)	pIJ1085	0.1	<1
IMA137 (*fix-137*::Tn5)	pIJ1085	0.1	<1
IMA16 (*fix-16*::Tn5)	pIJ1085	0.7	<1
IMA67 (*fix-67*::Tn5)	pIJ1085	0.6	<1
IMA37 (*fix-37*::Tn5)	pIJ1085	0.1	<1
IMA15 (*fix-15*::Tn5)	pIJ1085	0.3	<1
3688 (W.T.)	pIJ1085	100	<1

Acetylene reduction was measured 20 days after inoculating pre-germinated peas. In the absence of pIJ1086 or pIJ1085, the mutants gave values of acetylene reduction of <0.01% of the wild-type level.

Only low levels of acetylene reduction were found in plants inoculated with the *fix* mutant strains carrying plasmid pIJ1085, whereas relatively high levels of acetylene reduction were found using the other *fix* mutant strains carrying pIJ1086 (Table III). It seemed possible that the low levels of acetylene reduction may be due to the loss of plasmid pIJ1085 and so bacteria were isolated from the nodules and tested for their resistance to tetracycline. As shown in Table III, there is a correlation between the low levels of acetylene reduction and the loss of tetracycline resistance by the strains carrying plasmid pIJ1085. This instability appears to be specific to pIJ1085 since bacteria isolated from nodules infected with strains carrying pIJ1086 retained a relatively high proportion of tetracycline resistance when re-isolated from nodules. Although these two plasmids were lost at different frequencies following passage through nodules, no significant difference in their stability was found after growth in free living culture.

Discussion

Random Tn5 mutagenesis of plasmid pRL1JI (which is ~200 kb in size) resulted in the isolation of 12 symbiotically-defective mutants of *R. leguminosarum* (Ma *et al.*, 1982; Downie *et al.*, 1983). The mutant alleles span ~45 kb of DNA, thereby defining the region of pRL1JI involved in nodulation and symbiotic nitrogen fixation. In comparison, the genes involved in nitrogen fixation in *K. pneumoniae* span a region of ~23 kb of DNA.

It is not clear if the entire 45-kb region of pRL1JI is involved in symbiotic nitrogen fixation since there are two regions of DNA (~20 kb between the *fix-78*::Tn5 and *nod-57*::Tn5 alleles and ~6 kb between the *nod-128*::Tn5 and *fix-52*::Tn5 alleles) in which no *fix* or *nod* mutants were isolated in the primary screen. However, since only mutants completely defective in nodulation or nitrogen fixation were selected after mutagenesis of pRL1JI, it is possible that Tn5 insertions into these non-mutated regions may have given rise to inter-

mediate phenotypes such as delayed nodulation or reduced levels of nitrogen fixation.

The region of pRL1JI DNA represented in Figure 2 corresponds to that region of the symbiotic plasmid deleted in *R. leguminosarum* strain 6015, which has lost the *fix* and *nod* genes (Hirsch *et al.*, 1980; Hombrecher *et al.*, 1981). On the basis of genetic evidence, Buchanan-Wollaston *et al.* (1980) concluded that the *fix* and *nod* genes were closely linked. The results presented here show that in pRL1JI the *nod* genes lie between two closely-linked groups of *fix* genes. It has been established for *R. meliloti* (Banfalvi *et al.*, 1981; Rosenberg *et al.*, 1981; Long *et al.*, 1982; Kondorosi *et al.*, 1982), *R. trifoli* (Hooykaas *et al.*, 1981, Rolfe *et al.*, 1981) and *R. phaseoli* (Lamb *et al.*, 1982) that the *nod* and *fix* genes are linked but it has not yet been established if, in these species, the *nod* genes lie between *fix* genes.

Nitrogen fixation and nodulation ability could be restored to mutant strains by the appropriate plasmid (Table III) and it has been shown that either plasmid pIJ1085 or pIJ1089 could restore nodulation ability to the three nodulation-deficient mutants (Downie *et al.*, 1983). However, such studies may be of limited value with respect to genetic complementation for two reasons: (a) plasmid pIJ1085 segregates at a high level during the course of the plant growth; and (b) since recombination-deficient strains of *Rhizobium* are not available, recombination may occur and it is clearly difficult to assess the level of recombination which has occurred during growth of the strains within the nodule.

The availability of the cloned *fix* genes has made possible the identification of a region of pRL1JI DNA homologous to the *nifA* gene from *K. pneumoniae*. Nuti *et al.* (1979) and Ruvkun and Ausubel (1980) established that the nitrogenase genes of *K. pneumoniae* were homologous to those of *Rhizobium* (and other nitrogen-fixing bacteria), but did not detect homology with other *nif* genes, possibly because they were using *Rhizobium* genomic DNA rather than cloned fragments and thus had a less sensitive assay.

The *nifA* gene of *K. pneumoniae* activates transcriptional initiation of all the *nif* promoters in that strain (Dixon *et al.*, 1980; Buchanan-Wollaston *et al.*, 1981) except the *nifLA* operon which is under the control of the *ntrC* gene (Espin *et al.*, 1982; Drummond *et al.*, 1983). There appear to be close analogies between the *nifA* and *ntrC* genes in *K. pneumoniae* since the *nifA* gene product can substitute for the *ntrC* gene product (Merrick, 1983; Ow and Ausubel, 1983). Kennedy and Robson (1983) have shown that the cloned *K. pneumoniae nifA* gene can activate the *Azotobacter nif* genes, and it appears that the *R. meliloti nifH* promoter can be regulated by the *nifA* gene product from *K. pneumoniae* (Sunderesan *et al.*, 1983).

It is not yet clear if the regulation of nitrogen fixation genes in *Rhizobium* is similar to that in *K. pneumoniae*. The lack of observed homology between the *K. pneumoniae nifL* gene and DNA adjacent to the *nifA*-homologous region in *R. leguminosarum* may imply that the regulation is different, but it does appear that the activation of the nitrogen fixation genes may be similar and under the control of a *nifA*-like gene which is conserved among different species.

Materials and methods

Microbiological techniques

Bacterial strains and plasmids are listed in Table I. Media and general growth conditions were as described by Beringer (1974). The method for conjugal crosses was that of Buchanan-Wollaston *et al.* (1980). Peas (variety Wisconsin Perfection) were inoculated, grown and assayed for acetylene reduction according to Beynon *et al.* (1980). Nodules were routinely surface sterilised and the bacteria within the nodules isolated as described by Beringer (1974) and tested for tetracycline resistance by replica plating.

DNA isolation and manipulation

Plasmid DNA was isolated from *E. coli* strains by the method of Clewell and Helinski (1969). The cosmid gene bank was constructed essentially as described by Friedman *et al.* (1982) except that the DNA was size-fractionated on a 1.25 – 5 M NaCl salt gradient (made up in 10 mM Tris HCl pH 7.8, 1 mM EDTA) by centrifuging for 4 h at 140 000 *g* in a Beckman SW41 rotor.

DNA fragments were electrophoresed in gels containing 1% w/v agarose, and 40 mM Tris acetate pH 8.1. DNA was transferred to nitrocellulose filters as described by Southern (1975); DNA fragments were isolated from agarose gels as described by Smith (1980). Radioactively-labelled DNA was prepared by nick-translation (Rigby *et al.*, 1977).

Restriction enzymes were obtained from BRL (UK) Ltd. and used according to the manufacturers' instructions.

Electron microscopy of nodules

Root nodules were prepared for electron microscopy and thin sections were examined as described by Beringer *et al.* (1977).

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