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The *nodD* gene of *Rhizobium leguminosarum* is autoregulatory and in the presence of plant exudate induces the *nodA,B,C* genes

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To analyse *nod* gene expression in *Rhizobium leguminosarum*, a broad host-range *lacZ* protein fusion vector was constructed. Two protein fusions, *nodC-lacZ* and *nodD-lacZ*, were used to measure the regulation of expression of the promoters of the *nodA,B,C* and the *nodD* transcripts by measuring the induced levels of β-galactosidase activity in *R. leguminosarum*. In the absence of plant root exudate the *nodD-lacZ* hybrid was expressed but the *nodC-lacZ* hybrid was not. The expression of the *nodD-lacZ* hybrid was repressed in *R. leguminosarum* strains containing an intact cloned *nodD* gene indicating that the *nodD* gene is autoregulatory. The induction of the *nodC-lacZ* hybrid required both the *nodD* gene and a component present in plant root exudate. Therefore the *nodD* gene acts both as a repressor and as an activator of gene expression. The *nodD* gene is adjacent to *nodA* and transcribed divergently from *nodA,B,C* with only ~300 nucleotides between the coding regions of *nodA* and *nodD*. Within this intergenic region is a unique *BclI* site and, using *nodC-lacZ* or *nodD-lacZ* translational fusions with this *BclI* site as an end point, no induction of *nodC-lacZ* or *nodD-lacZ* was observed. Therefore the promoters of *nodD* and *nodA,B,C* overlap at least at this region, and the regulation of these overlapping promoters appears to be controlled by the *nodD* protein which becomes an activator only in the presence of a component from plant exudate.

Key words: lacZ fusion vector / nodulation / gene regulation / *Rhizobium*

Introduction

Early stages of the interaction between *Rhizobium* and a susceptible legume host plant involve a characteristic morphological event in which the plant root hairs curl back on themselves and appear to entrap the bacteria within the induced fold of the root hair. It is from within this fold that infection threads are often seen to develop; meristematic growth is induced by *Rhizobium* and the infection thread carries the bacteria into the meristem from which the nodule develops.

The bacterial genes involved in the induction of root hair curling have been located by Tn*5* mutagenesis on the symbiotic plasmids of a number of *Rhizobium* species, and close to these root hair curling genes other nodulation genes have also been identified (Kondorosi *et al.*, 1984; Downie *et al.*, 1985). In *R. leguminosarum* the nodulation and host-range genes lie within a 10-kb region of the symbiotic plasmid pRL1JI: two cosmid clones which overlapped by 10 kb were suffient to enable a strain of *R. phaseoli* cured of its symbiotic plasmid to nodulate peas rather than *Phaseolus* beans, the normal host of *R. phaseoli* (Downie *et al.*, 1983a).

Within this 10-kb region, four genes (*nodA,B,C,D*) were identified which were involved in the induction of root-hair curling. A strain deleted of its symbiotic plasmid but containing the cloned *nodA,B,C* genes could induce root-hair curling (Rossen *et al.*, 1984); a strain containing the *nodD*57::Tn*5* mutation on pRL1JI was significantly delayed in the induction of root-hair curling on peas and on the small seeded legume *Vicia hirsuta* (Downie *et al.*, 1985). The DNA sequence of the *nodA,B,C,D* region from both *R. leguminosarum* (Rossen *et al.*, 1984; Shearman *et al.*, in preparation) and *R. meliloti* (Torok *et al.*, 1984; Egelhoff *et al.*, 1985) has been determined. The *nodD* gene is transcribed divergently from the *nodA,B,C* genes in both species and there is a high degree of conservation of the *nodA,B,C,D* genes between the two species. Mutations in the *nodA,B,C* genes totally blocked nodulation in all species tested but mutations in *nodD* of *R. meliloti* gave a leaky phenotype (Jacobs *et al.*, 1985), sometimes nodulating alfalfa, whereas *nodD* mutations in *R. leguminosarum* and *R. trifolii* were Nod$^-$ (Downie *et al.*, 1985; Schofield *et al.*, 1983).

The biochemical roles of the *nod* genes have not been established. In order to study the regulation of the expression of these genes, a broad host-range vector was constructed, in which the *nod* genes could be fused to the *lacZ* gene from *Escherichia coli*. Using this vector in a Lac$^-$ strain of *R. leguminosarum* the regulation of these *nod* genes has been studied.

Results

Construction of pIJ1363 a broad-host-range lacZ protein fusion vector

Several gene fusion plasmid vectors involving the *lacZ* gene (β-galactosidase) of *E. coli* have been used for the detection and analysis of DNA sequences that contain transcriptional regulatory signals (Casadaban and Cohen, 1980). These vectors are particularly useful for analysis of the regulation of genes whose products are difficult to assay, but are limited in their application since they do not replicate in a wide variety of bacterial strains. To analyse the regulation of *R. leguminosarum nod* genes, a broad host-range vector was constructed containing the *E. coli lacZ,Y* genes cloned downstream of a transcriptional terminator.

The construction of pIJ1363 is described in Figure 1 and the relevant characteristics of the plasmid are: (i) it is based on the replicon pRK290 (derived from the P1-group, wide host-range plasmid RK2), is a low copy number plasmid and is 27 kb in size; (ii) the *lacZ,Y* genes are immediately downstream of a unique *Bam*HI site; (iii) upstream of the *Bam*HI site is the transcription terminator from bacteriophage *fd*, cloned as a 332-bp *Sau*3a fragment, one end of which constitutes half of the *Bam*HI site; (iv) genes cloned at the *Bam*HI site can form in-frame protein fusions with the eighth codon of *lacZ* and express β-galactosidase activity only under the control of their own promoter.

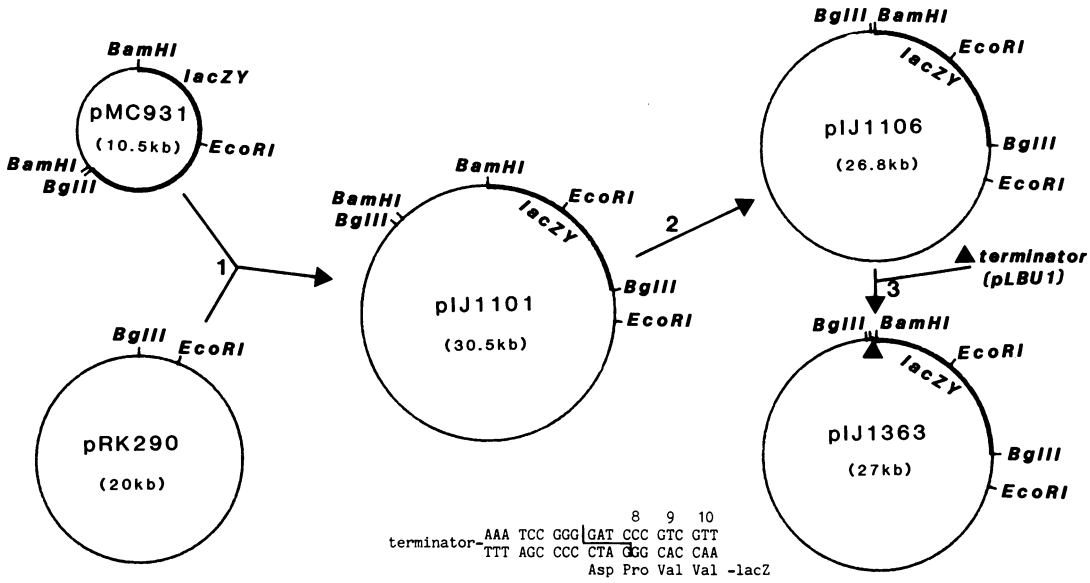

Fig. 1. 1. Plasmids pRK290 (Ditta *et al.*, 1980) and pMC931 (Casadaban and Cohen, 1980) were ligated at their unique *Bgl*II sites to form pIJ1101. **2.** Plasmid pIJ1101 was digested with *Bam*HI and re-ligated in order to eliminate the vector portion (pACYC177) of pMC931. The resultant plasmid pIJ1106 contains a unique *Bam*HI site. Genes cloned into this site can be fused to *lacZ* at the eighth codon but in preliminary experiments it was found that the level of transcription from a vector promoter was unacceptably high. **3.** In order to reduce this readthrough, the transcriptional terminator, originally from phage *fd* and cloned on pLBU1 (Beck *et al.*, 1978; Gentz *et al.*, 1981), was isolated as a 352-bp *Hind*III fragment. This fragment was digested with *Sau*3a and the 332-bp *Sau*3a fragment cloned into the *Bam*HI site of pIJ1106 to form pIJ1363. This step regenerated one *Bam*HI site since one end of the *Sau*3a fragment constitutes half of a *Bam*HI site. It was confirmed by restriction endonuclease mapping that the regenerated *Bam*HI site was adjacent to *lacZ* and that the terminator fragment was upstream of this *Bam*HI site. The DNA sequence around the *Bam*HI site including part of *lacZ* is presented.

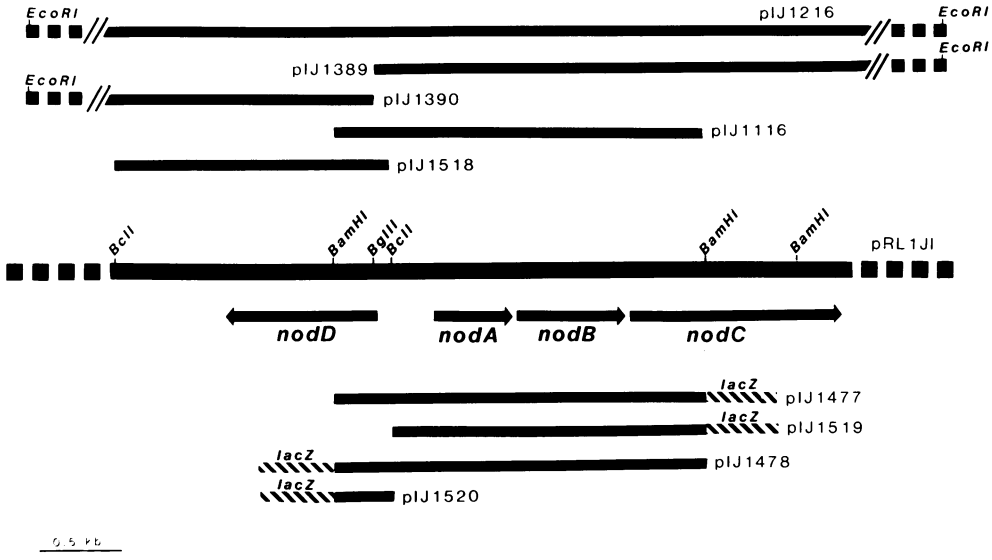

Fig. 2. The *nodA,B,C,D* region of *R. leguminosarum* plasmid pRL1JI showing the limits of the *nodA*, *nodB*, *nodC* and *nodD* coding regions as arrows. The fragments of DNA cloned into pIJ1363 and containing *nodC* and *nodD-lacZ* fusions are indicated in the lower half of the figure with the hatched line representing the *lacZ* gene. The fragments of DNA in the upper half of the figure were cloned into pKT230 and used in experiments to test for repression or induction activities (Table II). The restriction enzyme sites *Bcl*I, *Bam*HI, *Bgl*II and *Eco*RI were obtained from the DNA sequences (Rossen *et al.*, 1984; Shearman *et al.*, in preparation), and the sequences at the fusions are shown in Figure 3.

The nodD gene is autoregulatory

The 2.2-kb *Bam*HI fragment which extends from within *nodD* to within *nodC* was cloned into the *Bam*HI site of pIJ1363 to form pIJ1478 (Figure 2), and the 350-bp *Bam*HI-*Bcl*I fragment which extends from within *nodD* to the *Bcl*I site between *nodD* and *nodA* (Figure 2) was cloned into the *Bam*HI site of pIJ1363 to form pIJ1520. Plasmids pIJ1478 and pIJ1520 contain the *nodD* gene fused in-frame to the *lacZ* gene and the sequence of the *nodD-lacZ* junction is shown in Figure 3.

Plasmids pIJ1478 and pIJ1520 were transferred to the Lac$^-$ *Rhizobium* strain KH1122 which lacks a symbiotic plasmid. A significant level of β-galactosidase activity was observed with pIJ1478 but not with pIJ1520 (Table IA) indicating that in this recipient strain the *nodD* promoter is constitutively expressed and that the *nodD* promoter extends beyond the *Bcl*I site between *nodD* and *nodA* (Figure 2). The level of β-galactosidase activity observed with strain KH1122 containing pIJ1478 (*nodD-lacZ*) was reduced by ~60% if the symbiotic plasmid pRL1JI was pre-

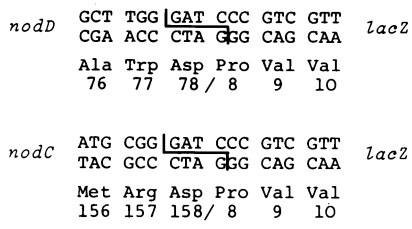

Fig. 3. DNA sequences at the *nodC-lacZ* and the *nodD-lacZ* protein fusions showing the junction at the *Bam*HI site (marked) plus the deduced amino acid sequences and codon numbers at the junctions of the protein fusions.

Table I. β-Galactosidase activities determined using the *nodD-lacZ* and *nodC-lacZ* fusions

Strain	Introduced plasmid (genes carried)	β-galactosidase activity	
		Minimal medium	Root exudate
A: *nodD-lacZ* fusions			
KH1122 pIJ1478	None	612 ± 146	627 ± 48
	pRL1JI (*nod*)	208 ± 8	204 ± 10
	pIJ1216 (*nodA,B,C,D,E*)	82 ± 31	n.t.
	pIJ1518 (*nodD*)	50 ± 27	36 ± 5
	pIJ1116 (*nodA,B*)	641 ± 8	n.t.
	pIJ1389 (*nodA,B,C*)	722 ± 28	n.t.
	pIJ1390 (*nodE*)	651 ± 47	n.t.
KH1122 pIJ1520	None	15 ± 4	n.t.
KH1122 pIJ1551	None	608 ± 32	n.t.
	pIJ1518 (*nodD*)	43 ± 12	n.t.
B: *nodC-lacZ* fusion			
KH1122 pIJ1477	None	47 ± 10	54 ± 12
	pRL1JI (*nod*)	53 ± 16	2413 ± 453
	pRL1JI*nodD*57::Tn*5*	70 ± 4	73 ± 13
	pIJ1518 (*nodD*)	56 ± 18	2431 ± 282
KH1122 pIJ1519	None	60 ± 16	75 ± 6
	pRL1JI (*nod*)	56 ± 18	81 ± 5
	pIJ1518 (*nodD*)	61 ± 17	87 ± 6

All assays were done, in duplicate, at least three times. No significant differences were found using different preparations of root exudate. n.t. = not tested.

sent. To determine if this repression was due to one of the *nod* genes, plasmids containing various *nod* genes were transferred to strain KH1122 (pIJ1478). Only those plasmids (pIJ1216 and pIJ1518 – see Figure 2) containing an intact *nodD* gene, inhibited the β-galactosidase activity showing that the *nodD* gene inhibits the expression of the *nodD-lacZ* fusion (Table IA).

It should be noted that pIJ1478 carries intact copies of the *nodA* and *nodB* genes (Figure 2); the *nodA,B* genes were deleted from pIJ1478 (using *Hind*III to delete an internal 1.7-kb fragment containing *nodA,B*; Rossen *et al.*, 1984) to form pIJ1551. As shown in Table IA, *nodD* is expressed constitutively with pIJ1551 and is repressed by pIJ1518, confirming that the *nodD* gene is autoregulatory and that no other pRL1JI *nod* genes are involved in its expression.

Regulation of nodC

The *Bam*HI site at codon 158 in *nodC* was used to make an in-frame protein fusion with the *lacZ* gene in pIJ1363. Two *nodC-lacZ* hybrid plasmids were constructed using this *Bam*HI site: pIJ1477 which extends from the *Bam*HI site in *nodD* and pIJ1519 which extends from the *Bcl*I site between *nodD* and *nodA* (Figure 2). The sequence of the *nodC-lacZ* fusion is shown in Figure 3.

Strong expression of *nodC-lacZ* was observed with pIJ1477 (Table IB) but only in the presence of root exudate and an intact copy of the *nodD* gene. The level of induction was similar irrespective of whether the *nodD* gene was present at a normal level (on pRL1JI) or cloned on a multicopy plasmid (pIJ1518). It is concluded that *nodD* was the only pRL1JI gene required for induction of the *nodC-lacZ* fusion on pIJ1477 since β-galactosidase activity was induced in the strain containing pIJ1518 (*nodD*) but lacking pRL1JI, and the induction of the *nodC-lacZ* fusion was blocked by the *nodD*57::Tn*5* mutation on pRL1JI.

In contrast, no induction of *nodC-lacZ* was observed with pIJ1519, showing that the *nodD*-activated *nodC* promoter extends beyond the *Bcl*I site between *nodD* and *nodA*.

Discussion

Four *R. leguminosarum* genes have been shown to be involved in the induction of normal root-hair curling (Rossen *et al.*, 1984. Downie *et al.*, 1985), the first observed step in nodulation. These nodulation genes form a cluster with *nodD* adjacent to *nodA* but transcribed divergently from *nodA,B,C* (Rossen *et al.*, 1984; Shearman *et al.*, in preparation). The interaction between rhizobia and leguminous plants first occurs on the root-hair and the interaction between plant and bacterium must involve precise regulation of bacterial and plant genes; overexpression of the *nodA,B,C* genes on a plasmid vector in a wild-type strain of *R. leguminosarum* prevented nodulation (Knight *et al.*, in preparation). Downie *et al.* (1985) observed that Tn*5* mutations in *nodD* significantly delayed the induction of root-hair curling and prevented nodulation. The results presented here show that the *nodD* gene is involved in the induction of the root-hair curling genes, *nodA,B,C*, and is itself autoregulatory.

The regulation of the *nod* genes was studied using a newly constructed broad host-range *lacZ* protein fusion vector, pIJ1363. Since this vector is based on the RK2 replicon it can be transferred to, and replicate in, a wide variety of Gram-negative bacteria and could be used for the study of gene regulation in these bacteria.

Using this vector it was established that a *nodD-lacZ* fusion (pIJ1478) was expressed constitutively in a strain which lacked an indigenous symbiotic plasmid. However, in strains containing a *nodD* gene, expression of the *nodD-lacZ* fusion was repressed. This repression was observed only with strains containing the intact *nodD* gene; overlapping subcloned fragments (Figure 2) containing the 5′ end (pIJ1116 and pIJ1389) or 3′ end (pIJ1390) of *nodD* had no effect on the expression of the *nodD-lacZ* fusion (pIJ1478), showing that the inhibition of transcription observed with pIJ1518 (*nodD*) was not due to competition between two promoters for a limiting activating factor. Therefore it can be concluded that in *R. leguminosarum* the *nodD* protein regulates expression at the *nodD* promoter.

Induction of *nodC-lacZ* required both an intact *nodD* gene and a component present in a fraction isolated from pea root exudate. Strong activation of *nodC-lacZ* (pIJ1477) was observed with strain KH1122 (which lacks an indigenous symbiotic plasmid) containing pIJ1518 (*nodD*) indicating that *nodD* is the only gene present on pRL1JI required for the induction of *nodC*. This was confirmed using a strain containing the *nodD*57::Tn*5* mutation on pRL1JI; in contrast to the normal wild-type strain, no induction of the *nodC-lacZ* hybrid was observed. Therefore, although pIJ1518 is a multicopy plasmid containing *nodD*, it is unlikely that the multiple copies of *nodD* abnormally induced the *nodC-lacZ* hybrid since the induction depended on root exudate and

Table II. Bacterial strains and plasmids

	Relevant characteristics	Reference
Rhizobium strains		
8401	*Rhizobium* strain cured of its symbiotic plasmid	Lamb *et al.*, 1982
KH1122	Tn*5*-containing mutant derivative of 8401; reduced β-galactosidase activity	This work
KH1122(pRL1JI)	As KH1122; but containing pRL1JI; nodulates normally on peas	This work
Plasmids		
pRL1JI	*R. leguminosarum* indigenous symbiotic plasmid	Johnston *et al.*, 1978)
pMC931	*lacZ*-protein fusion vector	Casadaban and Cohen, 1980
pRK290	Broad host-range cloning vector	Ditta *et al.*, 1980
pIJ1363	Broad host-range *lacZ*-protein fusion vector	This work
pIJ1477	*nodC-lacZ* derivative of pIJ1363	This work
pIJ1478	*nodD-lacZ* derivative of pIJ1363	This work
pIJ1519	*nodC-lacZ* derivative of pIJ1363	This work
pIJ1520	*nodD-lacZ* derivative of pIJ1363	This work
pIJ1551	*nodD-lacZ* derivtive of pIJ1363	This work
pKT230	Broad host-range cloning vector	Bagdasarian *et al.*, 1981
pIJ1116	2.2-kb *Bam*HI fragment containing *nodAB* region cloned in the *Bam*HI site of pKT230	This work
pIJ1216	6.6-kb *Eco*RI fragment cloned in pKT230	Downie *et al.*, 1985
pIJ1389	3.3-kb *Bgl*II-*Eco*RI *nodABC* fragment cloned in pKT230	Rossen *et al.*, 1984
pIJ1390	3.3-kb *Eco*RI-*Bgl*II *nod* fragment cloned in the *Bam*HI-*Eco*RI sites of pKT230	This work
pIJ1518	1.8-kb *Bcl*I *nodD* fragment cloned in the *Bam*HI site of pKT230 such that the *nodD* gene is expressed from a vector promoter	This work

a similar level was oberved with the strain containing only the native plasmid pRL1JI.

On the basis of the DNA sequence of the *nodA,B,C* genes (Rossen *et al.*, 1984), which showed only short intergenic regions, it was considered likely that these genes were within one transcriptional unit. This was confirmed by the finding that with pIJ1477 the *nodC*-promoter could be activated by *nodD* plus root exudate but with pIJ1519 no activation was observed. Therefore the promoter of *nodC* is on pIJ1477 and extends beyond (to the left as drawn in Figure 2) the *Bcl*I site between *nodD* and *nodA*, and it follows that *nodA,B* and *C* are within one operon.

The promoter of the *nodD* gene extends beyond (to the right as drawn in Figure 2) of this *Bcl*I site since pIJ1478 expressed the *nodD-lacZ* hybrid but pIJ1520 did not. Therefore the *nodD* and *nodA,B,C* promoters must overlap at least at the *Bcl*I site and it has been clearly demonstrated that the *nodD* gene represses one of these promoters (*nodD*) and, in the presence of root exudate, activates the other (*nodA,B,C*). Significantly, *nodD* repressed the expression of the *nodD-lacZ* fusion even in the presence of root exudate (Table IA), showing that if the *nodD* protein is converted to an 'activator' form by a component present in root exudate, it still represses the activation of *nodD*.

Mulligan and Long (1985), working on *R. meliloti*, also found that the *nodD* gene was required for the induction of the *nodA,B,C* genes in that species and that this required the presence of a factor from the exudate of alfalfa roots. In contrast, no autoregulation of *nodD* gene expression in *R. meliloti* was observed. The reason for this difference is not clear but it may be relevant to note that the phenotypes of *nodD* mutant strains in the two species are not the same. In *R. leguminosarum*, *nodD* mutations abolish nodulation completely (Downie *et al.*, 1985) but in *R. meliloti* such mutants only delay and reduce the numbers of nodules formed on alfalfa (Jacobs *et al.*, 1985).

The regulation of the *nodD, nodA,B,C* genes closely parallels the regulation of genes involved in arabinose utilisation by *E. coli*. In this system, *araC* is adjacent to *araB* and is transcribed divergently from *araB,A,D*; the *araC* protein is autoregulatory, inhibiting its own expression in the presence or absence of arabinose; the *araC* protein activates expression of *araB,A,D* but only in the presence of arabinose. The regulation of these *ara* genes is cyclic AMP-dependent and a model of their regulation and the location of protein binding domains within the overlapping *araC* and *araB,A,D* promoters has been described (Lee *et al.*, 1981).

The results presented here are analogous to those described for some virulence (*vir*) genes in *Agrobacterium tumefaciens* in which it has been shown that a plant-specified component was required for their induction (Stachel *et al.*, 1985). The relationship (if any) between the regulatory systems in these two genera remains to be determined.

At present, little is known of the biochemical basis of the special ability of *Rhizobium* to nodulate legumes. The finding that a plant-specified component is clearly required for the induction of *nod* genes offers the potential for the precise characterization of at least one of the steps involved in the symbiosis. It will be of importance to identify this factor and to establish if it has any other function in the nodulation process, over and above its role in *nod* gene regulation.

Materials and methods

Microbiological techniques

Bacterial strains and plasmids are listed in Table II or in the text. Media and general growth conditions were as described by Beringer (1974) and the method for conjugal crosses was that of Buchanan-Wollaston *et al.* (1980) except that the helper plasmid pRK2013 (Figurski and Helinski, 1979) was included in crosses containing derivatives of pKT230, pRK290 or pIJ1363 in order to mobilize these plasmids from *E. coli* to *R. leguminosarum*.

DNA isolation and manipulation

Restriction endonucleases, DNA ligase and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) were from Anglian Biotechnology Ltd. (UK). Plasmid DNA was isolated from *E. coli* as described by Maniatis *et al.* (1982) and from *Rhizobium* as described by Downie *et al.* (1983b). Restriction enzyme digests, DNA ligations and other general techniques were carried out as described by Maniatis *et al.* (1982).

Isolation of β-galactosidase-deficient mutant of R. leguminosarum

An *R. meliloti* Lac$^-$ mutant KS1 kindly supplied by E.Signer was complemented by selecting for growth on lactose following a conjugation using as donor, *E.*

coli containing a cosmid clone library of *R. leguminosarum* DNA (Downie *et al.*, 1983b). Plasmid DNA was prepared from one of the Lac^+ transconjugants, transferred to strain UNF510 (Merrick *et al.*, 1978) by transformation and then mutagenized with Tn*5* as described by Downie *et al.* (1985), selecting for transfer of kanamycin and tetracycline resistance to *Rhizobium*. Mutant derivatives of the cosmid clone were tested for their ability to complement strain KS1. One of the plasmids (pIJ1555) which did not complement the Lac^- defect was then transferred to *Rhizobium* strain 8401. The Tn*5* mutation on pIJ1555 was transferred to the chromosome of strain 8401 by marker exchange (Ruvkun and Ausubel, 1981) to form strain KH1122 which was white on agar plates containing X-gal, indicating that it had lost β-galactosidase activity. However, strain KH1122 was able to grow using lactose as a carbon source due to a second, low level, inducible β-galactosidase activity (J.P.W.Young, personal communication). This residual activity did not interfere with the β-galactosidase assays (Table I) and was not induced in the presence of plant root exudate under any conditions.

β-Galactosidase assays

The derivatives of *R. leguminosarum* were grown on Y minimal medium (Beringer, 1974) containing 0.2% mannitol as carbon source; 5 ml cultures in 25 ml screw cap bottles were shaken for 40 h at 28°C and the cells pelleted by centrifugation. The cell pellet was washed in 0.5 ml Z-buffer (Miller, 1972), pelleted by centrifugation and resuspended in 0.5 ml Z-buffer. Duplicate samples were diluted 1 in 10 in Z-buffer and the turbidity was measured at 600 nm using an MSE Spectroplus spectrophotometer. Cells were lysed with chloroform/SDS and 1 ml aliquots were assayed at 28°C for β-galactosidase using ortho-nitrophenyl-galactoside as described by Miller (1972). Before measuring the optical density at 420 nm the samples were cleared by centrifugation (1 min in an Eppendorf centrifuge). For those samples which had relative activities >250 units the cells were diluted a further 1 in 10 in Z-buffer before assaying. Units of activity were calculated as describd by Miller (1972).

In those experiments in which cells were grown in the presence of root exudate, mannitol (0.2%) and sodium glutamate (0.1%) were added to the root exudate and the cultures treated as described above.

Preparation of root exudate

Peas (variety Wisconsin perfection) were surface sterilized and aseptically germinated on agar plates as described previously (Beynon *et al.*, 1980). Four germinated peas were then added to 20 ml sterile FP medium (Fahraeus, 1957) and shaken in the dark in 250 ml flasks for 3 days. The medium was checked for contamination by plating a 0.1 ml sample onto a TY plate and incubating at 28°C for 2 days. Uncontaminated preparations were passed through a sterile Millipore filter and kept at −20°C for up to 4 weeks before use.

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