Enhanced Nitrogen Fixation and Competitiveness for Nodulation of *Lotus pedunculatus* by a Plasmid-cured Derivative of *Rhizobium loti*

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A plasmid-cured derivative of *Rhizobium loti* strain NZP2037, strain PN4010, was significantly more effective in N₂ fixation and showed an improved capacity to compete with other root nodule bacteria for nodulation of *Lotus pedunculatus*, relative to that of strain NZP2037. Reintroduction of the plasmid pRlo2037 into PN4010 resulted in a return to the NZP2037 level of symbiotic effectiveness and competitiveness. The enhanced effectiveness and competitiveness of PN4010 for *L. pedunculatus* was associated with its increased capacity to form nodules. Strain PN4010 did not differ from NZP2037 in its growth characteristics in pure culture, in its resistance to *L. pedunculatus* root flavolan or in its ability to multiply in the *L. pedunculatus* rhizosphere in the presence of a competing strain of *R. loti*. An examination of 35 *R. loti* strains (including 25 field isolates) revealed two strains (NZP2014 and NZP2042) that did not contain a single large indigenous plasmid; both were significantly more effective in N₂ fixation with *L. pedunculatus* than was NZP2037 or two other Nod⁺ Fix⁺ plasmid-containing strains of *R. loti*. Transfer of pRlo2037 into NZP2014 and NZP2042 reduced their effectiveness for *L. pedunculatus*.

INTRODUCTION

In *Rhizobium meliloti* and *R. leguminosarum* (which now includes the former species *R. phaseoli* and *R. trifolii*; Jordan, 1984), genes involved in root nodule development and N₂ fixation with legumes are located on large indigenous plasmids (reviewed by Dénarié *et al.*, 1981 and Kondorosi *et al.*, 1984). In contrast, these genes appear to be chromosomally located in the limited number of strains of *Bradyrhizobium* sp. that have been examined (Haugland & Verma, 1981; Masterson *et al.*, 1982). This difference in the location of nodulation (*nod*) and nitrogen fixation (*nif*) genes may reflect an evolutionary divergence between the two types of root-nodule bacteria (Pankhurst, 1986).

Rhizobium loti (Jarvis *et al.*, 1982) nodulates the agriculturally important pasture legume *Lotus* as well as a number of other legumes (Pankhurst, 1977; Jarvis *et al.*, 1982). A single large plasmid has been found in each of the two strains of *R. loti* (strains NZP2037 and NZP2213) so far examined (Pankhurst *et al.*, 1983). However, unlike the plasmids present in other *Rhizobium* species, those in strains NZP2037 and NZP2213 (pRlo2037 and pRlo2213 respectively) do not carry symbiotic genes (Pankhurst *et al.*, 1983; Chua *et al.*, 1985). Plasmid-cured derivatives of NZP2037 and NZP2213 still formed effective (N₂-fixing) root nodules on *Lotus* species (Chua *et al.*, 1985).

While testing *R. loti* strain NZP2037 and its plasmid-cured derivative strain PN4010 for symbiotic effectiveness on *Lotus pedunculatus* and *Lotus tenuis*, it was noted that PN4010 fixed noticeably more N₂ with *L. pedunculatus* than did NZP2037. We have sought to confirm this observation and to see if PN4010 acquired any other attributes, e.g. an improved capacity to compete with other *R. loti* strains for nodulation of *L. pedunculatus*, as a consequence of losing

plasmid pRlo2037. In addition we have screened a collection of wild-type *R. loti* strains for the presence of plasmids, with the aim of determining whether naturally occurring 'plasmid-minus' strains of *R. loti* exist. The symbiotic effectiveness of two such 'plasmid-minus' strains on *L. pedunculatus* and *L. tenuis* was evaluated.

METHODS

Bacterial strains and plasmids. These are listed in Table 1.

In a previous study, *R. loti* strain NZP2037 was cured of its 240 MDa plasmid (pRlo2037) by heat treatment of strain PN4005 (NZP2037 Str^R/pRlo2037::Tn5) (Chua *et al.*, 1985). A plasmid-cured derivative, strain PN4010, was subsequently obtained. In addition, a cointegrate between pRlo2037::Tn5 and pJB3JI was constructed (plasmid pPN360) and maintained in *Escherichia coli* strain PN440 (Chua *et al.*, 1985). The cointegrate plasmid pPN360 was transferred back into PN4010 to produce strain PN4012. Field isolates of *R. loti* were made from the nodules of *L. tenuis* plants collected from three different localities near Palmerston North, New Zealand. Each isolate was single-colony-purified and tested for its ability to form Nod⁺ Fix⁺ nodules on *L. tenuis* before it was used in the experiments.

Bacterial manipulations. *Rhizobium* and *Bradyrhizobium* cultures were grown at 28 °C in either M⁺ medium (Pankhurst, 1977), or S20 medium (Chua *et al.*, 1985) supplemented as required with neomycin (Neo; 400 µg ml⁻¹), rifampicin (Rif; 100 µg ml⁻¹), streptomycin (Str; 250 µg ml⁻¹) or tetracycline (Tc; 2 µg ml⁻¹). *E. coli* cultures were grown in LB medium (Miller, 1972), with tetracycline (15 µg ml⁻¹) and kanamycin (Km; 30 µg ml⁻¹) when necessary. Bacterial crosses were done as patch matings (Banfalvi *et al.*, 1981). Tests for flavolan resistance of rhizobia were as described by Pankhurst & Jones (1979).

Analysis of plasmids. Plasmids were detected in *Rhizobium* strains using a modification of the agarose gel method (Eckhardt, 1978) as described by Scott & Ronson (1982).

Symbiotic effectiveness. Three *Lotus* species, *L. pedunculatus* Cav. 'Grasslands Maku', *L. angustissimus* L. and *L. tenuis* Waldst. et Kit. were used to evaluate the symbiotic effectiveness (nodulation and N₂ fixation) of different *R.*

Table 1. *Bacterial strains and plasmids*

Strains and plasmids	Relevant characteristics	Source/reference		
<i>Rhizobium loti</i>				
NZP2014 NZP2037 NZP2042 NZP2085 NZP2241	Nod ⁺ Fix ⁺ (<i>L. pedunculatus</i> , <i>L. tenuis</i>)	DSIR culture collection		
NZP2146 NZP2213 NZP2238 NZP2260			Nod ⁺ Fix ⁺ (<i>L. tenuis</i>) Nod ⁺ Fix ⁻ (<i>L. pedunculatus</i>)	
PN4002 PN4005 PN4010 PN4012 PN4013 PN4200 PN4201 PN4204 PN4206 PN4225			NZP2037 Str ^R PN4002/pRlo2037::Tn5 PN4005 heat cured of pRlo2037::Tn5 PN4010/pPN360 PN4010/pJB3JI NZP2241 Rif ^R NZP2014 Str ^R NZP2042 Str ^R PN4201/pPN360 PN4204/pPN360	Pankhurst <i>et al.</i> (1983) Chua <i>et al.</i> (1985) This study
<i>Bradyrhizobium</i> sp. (<i>Lotus</i>)				
NZP2076 NZP2257 CC814s			Nod ⁺ Fix ⁺ (<i>L. pedunculatus</i>) Nod ⁺ Fix ⁻ (<i>L. tenuis</i>)	DSIR culture collection
<i>Agrobacterium tumefaciens</i>				
C58		C. Kado		
<i>Escherichia coli</i>				
PN440	HB101/pPN360	Chua <i>et al.</i> (1985)		
Plasmids				
pJB3JI pPN360	Km ^S derivative of R68.45 Ap ^R Tc ^R pRlo2037::Tn5::pJB3JI Ap ^R Km ^R Tc ^R	A. Johnston Chua <i>et al.</i> (1985)		

loti strains. The plants were grown under bacteriologically controlled conditions in jars of sterile pumice (Pankhurst & Jones, 1979) in a growth cabinet with a 12 h photoperiod (light intensity 180 W m²) with temperatures of 24 °C day, 20 °C night. At various times after inoculation with the test *Rhizobium*, plants from three to five replicate jars were removed and the roots washed free of pumice. The nodulated roots of individual plants were then blotted dry and their nitrogenase activity determined by acetylene reduction (Pankhurst & Jones, 1979). The nodules, roots and shoots of each plant were then dried overnight at 100 °C and weighed.

Rhizobium competition experiments. *L. pedunculatus* seedlings were grown on slopes of nitrogen-free agar medium in 15 × 1.5 cm test tubes (Pankhurst, 1981). Seedlings were inoculated with bacteria 3 d after they were sown in the tubes and the tubes were then placed in a growth cabinet. Bacterial cultures used for plant inoculation were grown on M⁺ agar for 2 to 4 d at 28 °C. The bacteria were scooped into 10 ml sterile water and the resultant suspensions adjusted to an OD₆₀₀ of 0.1 (about 1 × 10⁸ bacteria ml⁻¹) by the addition of extra water where necessary. Cultures were then mixed in equal proportion and 1 ml of each mixture was added to 12 *L. pedunculatus* seedlings. Seedlings inoculated with each test culture on its own were included as controls in each experiment. The number of viable bacteria in the inoculum was determined by plate counts made at the time of plant inoculation. The number of bacteria in the tubes at various times after inoculation was estimated as described by Pankhurst (1981). To identify bacteria in nodules, the latter were excised from the plant roots, surface-sterilized with a 1:1 (v/v) solution of 95% (v/v) ethanol and hydrogen peroxide for 1 min, rinsed 6 times with sterile water, crushed and their contents streaked onto M⁺ medium with or without addition of selective antibiotics. After incubation at 28 °C for 2 to 5 d the plates were examined for the presence or absence of bacterial growth.

RESULTS

Plasmid content and symbiotic effectiveness of NZP2037

To investigate the effect of pRlo2037 on the symbiotic effectiveness of NZP2037, strains PN4005, PN4010 and PN4012 were evaluated on *L. pedunculatus*, *L. angustissimus* and *L. tenuis* (Table 2). *L. angustissimus* was included in this experiment as it is similar to *L. pedunculatus* in its response to different *R. loti* strains (Pankhurst & Jones, 1979). Strain PN4010 was significantly more effective on *L. pedunculatus* and *L. angustissimus* than strains PN4005 or PN4012. After 6

Table 2. *Effect of R. loti* strains PN4005, PN4010 and PN4012 on the growth, nodulation and nitrogenase activity of three *Lotus* species

Plants were harvested and assayed 6 weeks after inoculation. The values for plant dry weight are means of 12 individual plants from four replicate jars (three plants per jar). All other values are means of nine individual plants from three replicate jars (three plants per jar). LSD, Least significant difference.

<i>Lotus</i> species and <i>R. loti</i> strain	Plant dry wt (mg)	No. of nodules	Nodule dry wt per plant (mg)	Acetylene reduction	
				μmol h ⁻¹ per plant	μmol h ⁻¹ per g nodule dry wt
<i>L. pedunculatus</i>					
PN4005	91.1	38.8	13.8	1.06	76.8
PN4010	147.6	64.0	25.4	2.12	83.4
PN4012	82.2	34.4	12.1	0.82	67.8
Uninoculated	16.5	0	0	0	0
LSD (<i>P</i> = 0.05)	11.6	12.3	3.7	0.36	11.9
<i>L. angustissimus</i>					
PN4005	85.2	55.3	22.0	1.48	67.3
PN4010	123.6	72.1	28.2	2.29	81.2
PN4012	67.8	51.4	17.5	1.11	63.4
Uninoculated	14.6	0	0	0	0
LSD (<i>P</i> = 0.05)	12.8	16.1	5.1	0.44	13.3
<i>L. tenuis</i>					
PN4005	182.8	20.7	13.7	1.26	92.0
PN4010	184.5	25.2	14.9	1.42	95.4
PN4012	171.5	19.3	12.0	0.91	75.8
Uninoculated	31.3	0	0	0	0
LSD (<i>P</i> = 0.05)	10.9	11.3	5.9	0.42	10.6

Fig. 1. Increases with time in plant dry weight (a), nodule dry weight (b) and acetylene reduction activity (c) of *L. pedunculatus* plants inoculated with strain PN4010 (●) or strain PN4005 (○). The vertical bar in each figure indicates the LSD (least significant difference; $P = 0.05$).

weeks growth *L. pedunculatus* and *L. angustissimus* plants inoculated with strain PN4010 had significantly more dry weight, nodules, nodule dry weight and nitrogenase activity per plant than plants inoculated with strain PN4005. However, the nitrogenase activity of the nodules formed by PN4010 and PN4005 on these two legumes was very similar (Table 2). Strain PN4012 exhibited a level of effectiveness comparable to that of PN4005 on both of these hosts. On *L. tenuis* there appeared to be no significant difference between the three strains in symbiotic effectiveness (Table 2). As controls to this experiment, no difference was observed between strains PN4005 and NZP2037 or between strains PN4010 and PN4013 (PN4010/pJB3JI) in symbiotic effectiveness on each of the three *Lotus* species.

Quantitative differences between strains PN4005 and PN4010 on *L. pedunculatus* (plant dry weight, nodule dry weight and plant nitrogenase activity) were measured over a 6 week growth period (Fig. 1). After 2 weeks, plants inoculated with strain PN4010 were more advanced in terms of total dry weight and nodule mass than plants inoculated with strain PN4005, and the differences became significant at 6 weeks. Both strains PN4005 and PN4010 caused root nodules to appear 10 d after inoculation.

Plasmid content and nodulation competitiveness of NZP2037

To test whether plasmid pRlo2037 could influence the nodulation competitiveness of strain NZP2037 the ability of strains PN4005, PN4010 and PN4012 to compete with other root nodule bacteria for nodulation of *L. pedunculatus* was examined. Each strain was tested against three other strains of *R. loti* and against three strains of *Bradyrhizobium* sp. (*Lotus*) (Table 3). The plasmid-cured strain PN4010 was a more efficient competitor than either of its plasmid-containing counterparts. This was particularly evident when *R. loti* strains NZP2241 and NZP2085 and *Bradyrhizobium* sp. (*Lotus*) strains NZP2257 and CC814s were competitors (Table 3). In competition with *R. loti* strain NZP2014, strain PN4010 performed only slightly better than strain PN4005 or PN4012.

One possible explanation for the enhanced competitiveness of strain PN4010 relative to that of PN4005 was that PN4010 bacteria achieved greater proportional representation in the rhizosphere in competition with the other strains than did strain PN4005. To test this, *L. pedunculatus* seedlings were inoculated with a low concentration (1×10^3 bacteria ml^{-1}) of PN4005 or PN4010 together with strain PN4200 (a rifampicin-resistant derivative of *R. loti* strain NZP2241). The number of PN4005, PN4010 and PN4200 rhizobia in the plant tubes was then followed over a 2 week period, until nodules were clearly visible. There was no apparent difference between strains PN4005 or PN4010 in their ability to grow in the presence of the competing strain PN4200 (data not shown).

Fig. 2. Agarose gel electrophoresis of plasmids in *R. loti* and the reference strain *A. tumefaciens* C58. Lane 1, *R. loti* NZP2037; lane 2, *R. loti* NZP2014; lane 3, *R. loti* NZP2085; lane 4, *R. loti* NZP2241; lane 5, *R. loti* NZP2042; lane 6, *R. loti* NZP2213; lane 7, *R. loti* NZP2260; lane 8, *R. loti* NZP2146; lane 9, *R. loti* NZP2238; lane 10, *A. tumefaciens* C58. Numbers indicate molecular size in MDa. Chr, chromosomal and linear DNA.

Table 3. Competition between *R. loti* strains PN4005, PN4010, PN4012 and other *R. loti* and *Bradyrhizobium* sp. (*Lotus*) strains for nodulation of *L. pedunculatus*

Five nodules from each of 12 6-week-old plants (total of 60 nodules) were tested for the presence of PN4005, PN4010 and PN4012 (streptomycin resistant). A minority (1 to 5%) of mixed nodules (containing both Str^R and Str^S bacteria) have not been included.

Competing strain	Percentage of nodules containing:		
	PN4005	PN4010	PN4012
<i>R. loti</i>			
NZP2014	32.5	57.5	28.7
NZP2085	43.7	81.2	33.7
NZP2241	55.0	100.0	21.5
<i>Bradyrhizobium</i> sp. (<i>Lotus</i>)			
NZP2076	25.0	41.8	18.7
NZP2257	28.7	83.7	16.2
CC814s	0.0	31.2	0.0

Growth rate and resistance to flavolans

The effect of plasmid pRlo2037 on the growth rate and sensitivity of strain NZP2037 to the *L. pedunculatus* root flavolan (Pankhurst & Jones, 1979) was examined. No difference in rates of growth of strains PN4005 and PN4010 in M⁺ or S20 medium could be detected: during exponential growth both strains had a doubling time of 5.5 h in M⁺ and 6.5 h in S20 medium. Similarly, no difference between the two strains in their sensitivity to the *L. pedunculatus* root flavolan was observed (data not shown).

Plasmid content and symbiotic effectiveness of field and laboratory strains of *R. loti*

To determine if differences in symbiotic effectiveness amongst wild-type *R. loti* strains for *Lotus* species was correlated with their plasmid content, a collection of 25 field isolates and nine laboratory strains (including strain NZP2037) were examined for plasmids. Using the Eckhardt lysis procedure, all of the field isolates and seven of the laboratory strains were found to contain a single large plasmid with a molecular weight of 120 to 270 MDa. Only two of the laboratory strains, NZP2014 and NZP2042 (Fig. 2, lanes 2 and 5) did not appear to contain a plasmid. This was confirmed in several repeat experiments.

The nine laboratory strains were each evaluated for their symbiotic effectiveness with *L. pedunculatus* and *L. tenuis* (Table 4). All formed N₂-fixing (Nod⁺ Fix⁺) nodules on *L. tenuis* and

Table 4. *Effect of R. loti strains on the dry weight of L. pedunculatus and L. tenuis plants*

Plants were harvested 6 weeks after inoculation. The figures given are the mean dry weight of 12 individual plants grown in four replicate jars (three plants per jar). LSD, Least significant difference.

<i>Rhizobium</i> strain	Presence of an indigenous plasmid*	<i>L. pedunculatus</i>		<i>L. tenuis</i>	
		Symbiotic phenotype	Plant dry wt (mg)	Symbiotic phenotype	Plant dry wt (mg)
NZP2014	—	Nod ⁺ Fix ⁺	112.2	Nod ⁺ Fix ⁺	167.9
NZP2037	+	Nod ⁺ Fix ⁺	88.6	Nod ⁺ Fix ⁺	159.1
NZP2042	—	Nod ⁺ Fix ⁺	105.5	Nod ⁺ Fix ⁺	150.3
NZP2085	+	Nod ⁺ Fix ⁺	52.1	Nod ⁺ Fix ⁺	148.2
NZP2241	+	Nod ⁺ Fix ⁺	74.7	Nod ⁺ Fix ⁺	161.4
NZP2213	+	Nod ⁺ Fix ⁻	12.9	Nod ⁺ Fix ⁺	173.4
NZP2146	+	Nod ⁺ Fix ⁻	13.0	Nod ⁺ Fix ⁺	141.3
NZP2238	+	Nod ⁺ Fix ⁻	14.1	Nod ⁺ Fix ⁺	182.5
NZP2260	+	Nod ⁺ Fix ⁻	11.8	Nod ⁺ Fix ⁺	160.8
Uninoculated			13.7		28.4
LSD ($P = 0.05$)			14.1		13.5

* Refer to Fig. 2.

there was little difference between the total dry weights of 6-week-old *L. tenuis* plants inoculated with each of these strains. On *L. pedunculatus*, five of the strains (NZP2014, NZP2037, NZP2042, NZP2085 and NZP2241) formed Nod⁺ Fix⁺ nodules while the remaining four formed ineffective (Nod⁺ Fix⁻) nodules. There was considerable difference in the total dry weight of 6-week-old *L. pedunculatus* plants inoculated with each of the five Nod⁺ Fix⁺ strains (Table 4): the two plasmid-minus strains (NZP2014 and NZP2042) had produced significantly more plant growth than the three plasmid-containing strains (NZP2037, NZP2085 and NZP2241). Transfer of pRlo2037 into strains NZP2014 and NZP2042 gave derivatives (PN4206 and PN4225 respectively) that were significantly less effective on *L. pedunculatus* than either parent strain. This was particularly marked with strain PN4206 which formed Nod⁺ Fix⁻ nodules on three out of six *L. pedunculatus* plants tested (data not shown).

DISCUSSION

The major result of this study was the observed increase in symbiotic effectiveness and competitiveness for *L. pedunculatus* shown by a plasmid-cured derivative of *R. loti* strain NZP2037. As reintroduction of the plasmid pRlo2037 (as plasmid pPN360) into the cured derivative (strain PN4010) resulted in a return to the NZP2037 level of symbiotic effectiveness and competitiveness for *L. pedunculatus*, the effects are clearly attributable to plasmid pRlo2037. This is the first report showing that an improvement in N₂-fixing ability and competitiveness can be achieved by removing an indigenous plasmid from a *Rhizobium* strain.

The enhanced symbiotic effectiveness shown by strain PN4010 for *L. pedunculatus* and *L. angustissimus* appeared to be associated with an increased production of nodules rather than with an increase in nodule size or nodule nitrogenase activity. This increased production of nodules did not result from nodules appearing earlier on the roots of plants inoculated with strain PN4010 relative to PN4005 but rather from the ability of the strain to initiate more nodules. The effect is similar to the increase in nodule production and N₂ fixation found for *R. meliloti* transconjugants harbouring the hairy root plasmid (pRi) of *Agrobacterium rhizogenes* (Strobel *et al.*, 1985). DeJong *et al.* (1981) also reported differences in the number of nodules produced by transconjugants of a *R. leguminosarum* strain harbouring different *R. leguminosarum* sym plasmids, although in this case increased nodule number did not result in increased N₂ fixation.

The lack of any significant improvement in effectiveness of strain PN4010 on *L. tenuis* may be related to the general capacity of *R. loti* strains to form Nod⁺ Fix⁺ nodules on this host. It is possible that the symbiotic potential of this symbiosis is already met by strain NZP2037. This is

not the case with *L. pedunculatus*, a host upon which *R. loti* strains of the NZP2037-type are still significantly less effective than the best *Bradyrhizobium* sp. (*Lotus*) strains (Pankhurst & Layzell, 1984; Wood *et al.*, 1985).

Associated with the enhanced symbiotic effectiveness shown by strain PN4010 for *L. pedunculatus* was a significant improvement in its ability to compete against other *R. loti* and *Bradyrhizobium* sp. strains for nodulation of *L. pedunculatus*. This improvement was not associated with any discernible difference between strains PN4010 and PN4005 in their respective abilities to grow in the presence of a competing strain in the *L. pedunculatus* rhizosphere. A similar result was obtained by Brewin *et al.* (1983) in a study of *sym* plasmid effects on the competitiveness of *R. leguminosarum*. We thus conclude that the enhanced competitiveness shown by strain PN4010 relative to strains PN4005 and PN4012 is associated with the improved 'infectivity' or capacity to form nodules on *L. pedunculatus*.

Two of 34 wild-type strains of *R. loti* examined did not contain a plasmid. As with strain PN4010, the absence of a plasmid in the strains NZP2014 and NZP2042 correlated with superior effectiveness for *L. pedunculatus*. In addition, strain NZP2014 competed equally with strain PN4010 in the competition study, suggesting that this strain may also be superior to other wild-type *R. loti* strains in competing for nodulation of *L. pedunculatus*. Transfer of pRlo2037 into the two wild-type strains NZP2014 and NZP2042 gave derivatives that were less effective in N₂ fixation on *L. pedunculatus* than either parent strain. This effect, which paralleled the reduction in effectiveness for *L. pedunculatus* found for PN4012 following re-introduction of pRlo2037, showed that pRlo2037 can suppress the nodulating capacity of strains other than NZP2037. As pRlo2037 does not carry genes directly involved in nodulation or N₂ fixation *per se* (Chua *et al.*, 1985) it will be of interest to elucidate the nature of the determinants that are responsible for this suppression, and to see if plasmids similar to pRlo2037 exist in other rhizobia. As most *Rhizobium* strains which contain a *sym* plasmid also contain other plasmids (Dénarié *et al.*, 1981), it is possible that interactions between the plasmids may be equally significant in determining the success of the symbiosis.

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