Journal of General Microbiology (1981), **124**, 1–7. *Printed in Great Britain*

Effects of Plasmid Content in *Rhizobium leguminosarum* on Pea Nodule Activity and Plant Growth

By T. M. DEJONG,[1]* N. J. BREWIN[2] AND D. A. PHILLIPS[1]

[1]*Department of Agronomy and Range Science, University of California, Davis, California 95616, U.S.A.*

[2]*John Innes Institute, Colney Lane, Norwich NR4 7UH*

(*Received 19 August 1980; revised 27 October 1980*)

Pea plants that were nodulated by *Rhizobium leguminosarum* strains 248, 300 or 3622 and grown in the absence of combined nitrogen differed significantly in dry weight, leaf area, nitrogen content, nodule mass and nodule number. Transfer of plasmids from these field isolates to strain 16015, a non-nodulating derivative of strain 300, resulted in new strains capable of both nodulation and N_2 fixation. In plants nodulated by these new strains the numbers of root nodules were significantly different, but there were no significant differences in N_2 fixation, leaf area or dry weight. In several cases the introduction of additional plasmids into strain 300 or strain 16015 impaired symbiotic performance relative to strain 300 itself. Of all plant traits measured in symbiotic associations, leaf area was most highly correlated with the total Kjeldahl nitrogen content of plants after 25 d growth in the absence of combined nitrogen.

INTRODUCTION

At least some genetic information responsible for both root infectivity and nodule effectiveness is located on plasmids in *Rhizobium* species (Higashi, 1967; Dunican *et al.*, 1976; Johnston *et al.*, 1978; Nuti *et al.*, 1979; Brewin *et al.*, 1980*a*, *b*). Such reports have generally been qualitative because the presence or absence of a phenotype was examined. No quantitative data on actual N_2 fixation and plant growth are available for *Rhizobium*–legume symbioses involving *Rhizobium* strains altered by plasmid transfer. As positive genetic factors that influence symbiotic N_2 fixation in *Rhizobium* are identified, the quantitative effect of such factors on the symbiosis and any consequences of plasmid content *per se* become important.

In the studies described in this report various combinations of plasmid-linked genes were transferred into a common genetic background so that the physiological effects associated with plasmid content could be investigated. The plasmids, which were all derived from strains of *Rhizobium leguminosarum*, were transferred into the *R. leguminosarum* field isolate, strain 300, or into strain 16015, a non-nodulating derivative of strain 300 which has suffered a deletion in certain plasmid-linked genes (Hirsch *et al.*, 1980), including genes concerned with nodule formation (*nod*) and nodule function (*fix*) (Buchanan-Wollaston *et al.*, 1980). Quantitative measurements were made of nodulation characteristics and host–plant responses of Alaska peas (*Pisum sativum* L.) inoculated with these genetically altered strains of *R. leguminosarum*. The aims of the study were: (1) to provide a functional analysis of a series of *R. leguminosarum* strains that differed only in their plasmid content; (2) to determine if the insertion of genetic markers for *medium* bacteriocin production or kanamycin resistance into the *R. leguminosarum* genome was necessarily associated with changes in symbiotic effectiveness; (3) to compare various measurements of *R. leguminosarum* effectiveness so that future screening of *Rhizobium* mutants for symbiotic effectiveness might be simplified.

0022-1287/81/0000-9504 $02.00

Table 1. *Characteristics of Rhizobium leguminosarum strains studied*

R. leguminosarum strain	Symbiotic phenotype*	Characters	Reference
16015	Nod^-	300 *str-37, spc-54, nod-6007*	Brewin *et al.* (1980*a*)
248	$Nod^+ Fix^+$	Field isolate	Hirsch (1979)
3747	$Nod^+ Fix^+$	16015 pRL1JI	Brewin *et al.* (1980*a*)
3738	$Nod^+ Fix^+$	16015 pVW1JI	Brewin *et al.* (1980*a*)
300	$Nod^+ Fix^+$	Field isolate	Johnston & Beringer (1975)
3745†	$Nod^+ Fix^+$	16015 pRL4JI	Brewin *et al.* (1980*a*)
3740†	$Nod^+ Fix^+$	16015 pVW5JI	Brewin *et al.* (1980*a*)
3622	$Nod^+ Fix^+$	Field isolate	Brewin *et al.* (1980*b*)
3737	$Nod^+ Fix^+$	16015 pRL5JI	Brewin *et al.* (1980*b*)
3711	$Nod^+ Fix^+$	300 pRL3JI	This study
3713	$Nod^+ Fix^+$	300 pRL1JI	This study
3718	$Nod^+ Fix^+$	300 pJB5JI	This study

* Nod^+ and Fix^+ refer to nodulation and N_2 fixation functions that are absent from *R. leguminosarum* strain 16015.

† These strains acquired the Nod^+ determinants from strain 300, presumably as a result of recombination with pRL4JI or pVW5JI prior to transfer to strain 16015.

Table 2. *Characteristics of Rhizobium leguminosarum plasmids studied*

Plasmid	Source	Phenotypic characters*	Reference
pRL1JI	Strain 248	$Med^+ Nod^+ Fix^+ Tra^+$	Hirsch (1979)
pRL3JI	Strain 306	$Med^+ Tra^+$	Hirsch (1979)
pRL4JI	Strain 309	$Med^+ Tra^+$	Hirsch (1979)
pRL5JI	Strain 3622	$Nod^+ Fix^+ Tra^+$	Brewin *et al.* (1980*b*)
pJB5JI	pRL1JI::Tn5†	Med^- Kan-r $Nod^+ Fix^+ Tra^+$	Johnston *et al.* (1978)
pVW1JI	pRL1JI::Tn5	Med^- Kan-r $Nod^+ Fix^+ Tra^+$	Brewin *et al.* (1980*a*)
pVW5JI	pRL4JI::Tn5	Med^- Kan-r Tra^+	Brewin *et al.* (1980*a*)

* Med^+ means that the plasmid specifies *medium* bacteriocin; Nod^+ and Fix^+ refer to nodulation and N_2 fixation functions that are absent from *R. leguminosarum* strain 16015; Tra^+ means transmissible by conjugation; Kan-r indicates kanamycin resistance (60 μg ml^{-1}).

† pRL1JI::Tn5 means that transposon Tn5 was inserted into the region determining production of *medium* bacteriocin (Med) of pRL1JI.

METHODS

Rhizobium leguminosarum strains and plasmids. Strains used are listed in Table 1 and plasmids transferred in Table 2.

Bacterial manipulations. Conditions for bacterial culture were as described by Beringer (1974). Membrane crosses were done as described by Beringer *et al.* (1978). The production of *medium* bacteriocins was tested as described by Hirsch (1979), using *R. leguminosarum* strain 336 as the indicator.

Plant inoculations. Each *R. leguminosarum* strain was used to inoculate six plants. At the conclusion of every experiment bacteria from four randomly selected nodules on each plant were re-isolated. The identity of the nodule isolates was confirmed by the intrinsic drug-resistance patterns (Josey *et al.*, 1979), by the production of *medium* bacteriocin, or by electrophoretic patterns on agarose gels (Hirsch *et al.*, 1980).

Plant culture. Alaska pea (*Pisum sativum* L.) plants were grown in a controlled environment chamber under a 14 h/10 h light/dark cycle at 21/20 °C, 50% relative humidity and a photosynthetic photon flux density of 650 μE m^{-2} s^{-1}. Plants were grown individually in modified 'Leonard jar' assemblies (Leonard, 1943) consisting of a bottomless, 750 ml wine bottle that was inserted neck-first into a 1 litre glass jar. The neck of the wine bottle was secured with a two-hole rubber stopper and filled with vermiculite and a 1 cm thick surface layer of perlite. The jar acted as a nutrient solution reservoir and initially contained 750 ml of N-free nutrient solution. The nutrient solution contained 2 mM-$CaSO_4$, 1 mM-K_2SO_4, 1 mM-K_2HPO_4, 2 mM-$MgSO_4$, 4 μM-$CoCl_2$, micronutrient solution (Johnson *et al.*, 1957) at 1 ml l^{-1}, and Sequestrene 138Fe iron chelate (Ciba-Geigy) at 18·7 mg l^{-1}. The pH of the solution was adjusted to pH 7·0 with HCl; it changed by less than 0·5 pH units during the course of each

Table 3. *Effects of Rhizobium leguminosarum strains on root nodule activity*

Strain 16015 formed no nodules and therefore had no root nodule activities. Apparent N_2 fixation was calculated as (acetylene reduced − H_2 evolved in air)/3.

R. leguminosarum strain	Acetylene reduction per plant (μmol h^{-1})	H_2 evolution per plant (μmol h^{-1})	Apparent N_2 fixation per plant (μmol h^{-1})
248	9·59	6·10	1·15
3747	11·12	6·24	1·63
3738	12·42	6·08	2·11
300	14·06	8·27	1·93
3745	11·05	3·84	2·37
3740	12·98	6·92	2·02
3622	6·20	1·87	1·44
3737	11·95	5·38	2·19
3711	11·61	3·16	2·85
3713	11·73	4·71	2·34
3718	10·26	5·20	1·69
L.S.D.* (0·05)	2·22	0·96	0·60

* Least significant difference.

experiment. The complete Leonard jar assemblies were autoclaved prior to planting. The sides of the assemblies were covered with aluminium foil to prevent light absorption. Pea seeds weighing 0·21–0·23 g were surface-sterilized with 70% (v/v) ethanol, rinsed and germinated on sterile paper towels moistened with distilled water. After 3 d of imbibition seedlings were selected for uniformity, planted in the sterile Leonard jar assemblies, and inoculated with one of the *R. leguminosarum* strains (Table 1).

Measurements of symbiotic associations. All data were collected from 25-d-old plants. Acetylene-dependent ethylene production (referred to less rigorously as acetylene reduction), H_2 evolution in air, and $^3H_2$ incorporation in air/acetylene/tritiated H_2 (0·88:0·10:0·02, by vol.) were measured on excised root systems (Bethlenfalvay & Phillips, 1977, 1979). Leaf area was determined with an area meter (LI-3000; Lambda Instruments, Lincoln, Nebr., U.S.A.). Plant dry weight was measured after 48 h at 75 °C, and N content was determined by Kjeldahl analysis (Burris & Wilson, 1957).

RESULTS

Effect of plasmid transfer to strain 16015

The three field isolates (strains 248, 300 and 3622) showed distinct strain effects on two short-term measures of root nodule function, acetylene reduction and H_2 evolution (Table 3), and on actual plant N content (Table 4). The strain effects on those parameters were highly significant ($P \leqslant 0{\cdot}01$). Such results, however, were not observed in root nodules containing strains produced by transferring plasmids from strains 248, 300 or 3622 to the non-nodulating strain 16015. In these strains (3747, 3738, 3745, 3740 and 3737), which presumably contained nodulation determinants from the various field isolates, there was no significant difference in total electron flow through the nitrogenase enzyme complex, measured as acetylene reduction, and only strain 3745 showed significantly different ($P \leqslant 0{\cdot}05$) H_2 evolution in air (Table 3). Total Kjeldahl N values of plants nodulated by the various *R. leguminosarum* strains and grown with N-free nutrient solution supported the conclusion that strains 3747, 3738, 3745, 3740 and 3737 fixed equivalent amounts of N_2 during the 25 d growth period (Table 4). By subtracting the N content of plants inoculated with the non-nodulating strain 16015 from the total N content of nodulated plants, one can calculate that these strains reduced an average of 14·7 mg N_2 while the field isolates 248, 300 and 3622 supplied 16·6, 24·0 and 3·7 mg N, respectively, from N_2.

None of the root nodules formed by strains listed in Table 1 incorporated $^3H_2$ at significant rates, suggesting that all these strains lacked the determinants necessary to express the uptake

Table 4. *Effects of Rhizobium leguminosarum strains on plant growth and nodulation*

R. leguminosarum strain	Plant dry weight (g)	Plant N content (mg)	Leaf area (cm^2)	Nodule dry weight per plant (mg)	Nodule number per plant
16015	0·527	8·9	46·6	—	—
248	0·640	25·5	80·6	30·4	134
3747	0·561	23·6	72·5	41·8	145
3738	0·606	23·4	75·6	43·3	173
300	0·758	32·9	102·5	54·5	266
3745	0·608	23·1	77·6	38·4	392
3740	0·656	25·2	83·8	37·7	275
3622	0·500	12·6	50·0	23·6	314
3737	0·578	22·8	75·0	52·0	322
3711	0·607	23·7	74·7	38·7	268
3713	0·792	32·4	101·5	41·7	225
3718	0·677	29·1	93·4	43·6	209
L.S.D.* (0·05)	0·095	4·7	11·9	6·5	54

* Least significant difference.

hydrogenase system (i.e. Hup^-). The positive control, Hup^+ *R. leguminosarum* strain 128C53, incorporated three orders of magnitude more $^3H_2$ in similar assays (data not shown). Thus, the absence of an uptake hydrogenase by this criterion permitted a calculation of apparent N_2 fixation (Table 3) as (acetylene reduced $- H_2$ evolved in air)/3 using assumptions discussed by Burns & Hardy (1975). Field isolates 248 and 300 differed significantly ($P \leqslant 0{\cdot}05$) for apparent N_2 fixation while bacteria produced from strain 16015 by plasmid transfer gave generally equivalent rates.

Varying N_2 fixation (i.e. plant N content) by field isolates 248, 300 and 3622 was reflected by highly significant differences ($P \leqslant 0{\cdot}01$) in dry weight and leaf area of plants nodulated by those strains (Table 4). Plants nodulated by strains 3747, 3738, 3745, 3740 or 3737 were not significantly different ($P \leqslant 0{\cdot}05$) for these parameters.

Although N_2 fixation parameters and resulting plant growth were similar for bacteria produced from strain 16015 by plasmid transfer from field isolates 248, 300 or 3622, the capability of these strains to form root nodules differed significantly (Table 4). The number of root nodules per plant, one measure of nodulation capability as opposed to N_2 fixation activity, differed significantly ($P \leqslant 0{\cdot}05$) among field isolates 248, 300 and 3622, but in four out of five cases, strains generated by transferring plasmids from these field isolates into strain 16015 formed a similar number of nodules to the field isolate from which plasmids had been mobilized (Table 4). In the fifth case, strain 3745, significantly more root nodules ($P \leqslant 0{\cdot}05$) were formed than with the corresponding field isolate or any other strain in this study.

Effect of plasmid transfer to strain 300

Mobilizing plasmids into strain 16015 did not permit any measure of the effect of plasmid content *per se* because strain 16015 formed no root nodules and thus could not reduce N_2 (Table 4). Transferring plasmids pRL1JI, pRL3JI or pJB5JI into field isolate 300 resulted in diverse effects on nodulation and N_2 fixation functions (Tables 3 and 4). Plasmid pRL1JI had no significant effect in strain 3713 on the actual plant N content or number of root nodules per plant compared with strain 300, but pRL3JI and pJB5JI in strains 3711 and 3718, respectively, decreased either the plant N content or the number of root nodules formed (Table 4). In no case was nodulation or N_2 fixation increased relative to strain 300 by plasmid transfer. Short-term measures of root nodule activity for strains 3711, 3713 and 3718 (Table 3) were not reflected in actual N accumulation data (Table 4).

Table 5. *Correlation between total Kjeldahl nitrogen accumulation and various measures of plant growth and nodule activity*

Data were calculated from 14 mean values, each derived from six replicate plants nodulated by a specific strain of *Rhizobium*. Strains TA101 and 92F1 (Bethlenfalvay *et al.*, 1978) were used in addition to the 12 strains listed in Table 1 in order to provide a wide range of N_2 fixation abilities. Average Kjeldahl N contents of 25-d-old plants from the 14 groups were tested for a possible correlation with each factor listed.

Factor	Correlation (r^2)
Leaf area	0·963
Total plant dry weight	0·816
Acetylene reduction	0·713
Nodule dry weight	0·636
Apparent N_2 fixation*	0·463
Nodule number	0·075

* Apparent N_2 fixation was calculated as (acetylene reduced − H_2 evolved in air)/3.

Indirect assessment of N_2 fixation by Rhizobium strains

Actual N_2 fixation, measured by Kjeldahl N accumulation, in Alaska peas grown under uniform conditions in the absence of combined N was correlated most highly ($r^2 = 0·963$) with total leaf area (Table 5). Other indirect measures of N_2 fixation were less well correlated with N content.

DISCUSSION

Quantitative measurements of root nodule formation and N_2 fixation in *Rhizobium*–pea symbioses showed that although strain 16015, a plasmid deletion mutant of strain 300 lacking genes for nodule formation (Hirsch *et al.*, 1980; Buchanan-Wollaston *et al.*, 1980), became capable of forming root nodules after receiving plasmid-borne determinants for nodulation ability from field isolates 248, 300 or 3622, the amount of N_2 reduced was the same regardless of the plasmid transferred (Tables 3 and 4). The same derivatives of strain 16015, however, did produce different numbers of root nodules (Table 4). Because the field isolates 248, 300 and 3622 differed significantly in both the number of nodules formed and the amount of N_2 fixed (Tables 3 and 4), the results suggest that genetic determinants transferred by plasmids pRL1JI, pVW1JI, pRL4JI, pVW5JI and pRL5JI were responsible for suppression of the lesion affecting nodule formation in strain 16015, whereas determinants present (but previously unexpressed because no nodules were formed) within strain 16015 were responsible for the similar N_2 fixation abilities of all these transconjugants.

A comparison of strains 300, 3711, 3713 and 3718 shows that plasmid transfer itself did not necessarily enfeeble or strengthen symbiotic properties of strain 300 (Table 4). The fact that strain 3713 contained pRL1JI and reduced as much N_2 as strain 300 suggests that potentially favourable traits such as bacteriocin production might be transferred to a particular strain without altering N_2 fixation. However, because the presence of pRL3JI and pJB5JI in strains 3711 and 3718, respectively, decreased N_2 fixation and root nodule formation compared with strain 300, recipients of new plasmids will have to be monitored carefully. It should be noted in passing that strains 3713 and 3718 probably contain duplicates of some symbiotic genes compared with strains 3747 and 3738 because pRL1JI and pJB5JI do not necessarily eliminate resident plasmids when transferred to derivatives of strain 300 (Hirsch *et al.*, 1980). The fact that strains 3713 and 3718 reduced more N_2 is suggestive in this regard, although neither 3713 nor 3718 reduced more N_2 than strain 300 itself. A more complete series of comparative strains will be required before any definite conclusion can be drawn about any possible effect of gene dosage.

Some controversy exists over the relationship between antibiotic resistance and *Rhizobium* effectiveness (Schwinghamer, 1964, 1967; Pankhurst, 1977; Hagedorn, 1979; Bromfield &

Jones, 1979). Schwinghamer (1967) reported no correlation between spontaneous kanamycin resistance and symbiotic effectiveness for *R. leguminosarum*. In the present study several of the strains were kanamycin-resistant because they contained an introduced transposon Tn5-coded drug inactivation system (Berg *et al.*, 1975). From the limited number of comparisons that could be made, there was no evidence of a correlation between transposon-determined kanamycin resistance and symbiotic effectiveness, although unfortunately comparisons were confounded by parallel changes in *medium* bacteriocin production. Neither strains 3747 and 3738 nor 3745 and 3740 showed significant differences in symbiotic N_2 fixation (Tables 3 and 4).

One very significant finding in this study is the observation that total Kjeldahl N content of Alaska peas grown for 25 d on N-free nutrient solution was highly correlated with leaf area (Table 5). The high correlation probably reflects the fact that leaves are a very large sink for N in young legumes (Pate *et al.*, 1979). Indirect measures of N_2 fixation such as plant dry weight, acetylene reduction, or apparent N_2 fixation, which combines acetylene reduction and H_2 evolution, were less well correlated with actual N_2 fixation. In some cases, such as strains 3711, 3713 and 3718, the short-term measures of N_2 fixation (Table 3) were misleading when compared with the close relationship between leaf area and N content (Table 4). Although the most accurate measure of N_2 fixation is total N accumulation by Kjeldahl analysis for peas growing under uniform conditions in the absence of combined N, that method may not be the most convenient for evaluating symbiotic proficiency among a variety of genetically altered *Rhizobium* strains. The fact that, of the parameters reported in Table 5, leaf area is the quickest and easiest to measure suggests that in the future this trait could be useful for assessing symbiotic effectiveness of genetically altered *Rhizobium* strains.

This research was supported in part by NATO Research Grant 1533 and National Science Foundation Grants PFR 77-07301 and PCM 78-01146. We acknowledge the technical assistance of S. S. Jue and E. A. Wood, and we thank Dr A. W. B. Johnston and Professor D. A. Hopwood for their criticisms of the manuscript.

REFERENCES

BERG, D. E., DAVIES, J., ALLET, B. & ROCHAIX, J. (1975). Transposition of R factor genes to bacteriophage λ. *Proceedings of the National Academy of Sciences of the United States of America* **72**, 3628–3632.

BERINGER, J. E. (1974). R factor transfer in *Rhizobium leguminosarum*. *Journal of General Microbiology* **84**, 188–198.

BERINGER, J. E., HOGGAN, S. A. & JOHNSTON, A. W. B. (1978). Linkage mapping in *Rhizobium leguminosarum* by means of R plasmid-mediated recombination. *Journal of General Microbiology* **104**, 201–207.

BETHLENFALVAY, G. J. & PHILLIPS, D. A. (1977). Ontogenetic interactions between photosynthesis and symbiotic nitrogen fixation in legumes. *Plant Physiology* **60**, 419–421.

BETHLENFALVAY, G. J. & PHILLIPS, D. A. (1979). Variation in nitrogenase and hydrogenase activity of Alaska pea root nodules. *Plant Physiology* **63**, 816–820.

BETHLENFALVAY, G. J., ABU-SHAKRA, S. S. & PHILLIPS, D. A. (1978). Interdependence of nitrogen nutrition and photosynthesis in *Pisum sativum* L. II. Host plant response to nitrogen fixation by *Rhizobium* strains. *Plant Physiology* **62**, 131–133.

BREWIN, N. J., BERINGER, J. E., BUCHANAN-WOLLASTON, A. V., JOHNSTON, A. W. B. & HIRSCH, P. R. (1980*a*). Transfer of symbiotic genes with bacteriocinogenic plasmids in *Rhizobium leguminosarum*. *Journal of General Microbiology* **116**, 261–270.

BREWIN, N. J., BERINGER, J. E. & JOHNSTON, A. W. B. (1980*b*). Plasmid-mediated transfer of host range specificity between two strains of *Rhizobium leguminosarum*. *Journal of General Microbiology* **120**, 413–420.

BROMFIELD, E. S. P. & JONES, D. G. (1979). The competitive ability and symbiotic effectiveness of doubly labelled antibiotic resistant mutants of *Rhizobium trifolii*. *Annals of Applied Biology* **91**, 211–219.

BUCHANAN-WOLLASTON, A. V., BERINGER, J. E., BREWIN, N. J., HIRSCH, P. R. & JOHNSTON, A. W. B. (1980). Identification of symbiotically defective mutants by insertion of the transposon Tn5 into a transmissible plasmid. *Molecular and General Genetics* **178**, 185–190.

BURNS, R. C. & HARDY, R. W. F. (1975). *Nitrogen Fixation in Bacteria and Higher Plants*, pp. 121–122. New York, Heidelberg & Berlin: Springer-Verlag.

BURRIS, R. H. & WILSON, P. W. (1957). Methods for measurement of nitrogen fixation. *Methods in Enzymology* **4**, 355–366.

DUNICAN, L. K., O'GARA, F. & TIERNEY, A. B. (1976). Plasmid control of effectiveness in *Rhizobium*: transfer of nitrogen-fixing genes on a

plasmid from *Rhizobium trifolii* to *Klebsiella aerogenes*. In *Symbiotic Nitrogen Fixation in Plants*, pp. 77–90. Edited by P. S. Nutman. Cambridge: Cambridge University Press.

HAGEDORN, C. (1979). Relationship of antibiotic resistance to effectiveness in *Rhizobium trifolii* populations. *Soil Science Society of America Journal* **43**, 921–925.

HIGASHI, S. (1967). Transfer of clover infectivity of *Rhizobium trifolii* to *Rhizobium phaseoli* as mediated by an episomic factor. *Journal of General and Applied Microbiology* **13**, 391–403.

HIRSCH, P. R. (1979). Plasmid-determined bacteriocin production by *Rhizobium leguminosarum*. *Journal of General Microbiology* **113**, 219–228.

HIRSCH, P. R., VAN MONTAGU, M., JOHNSTON, A. W. B., BREWIN, N. J. & SCHELL, J. (1980). Physical identification of bacteriocinogenic, nodulation and other plasmids in strains of *Rhizobium leguminosarum*. *Journal of General Microbiology* **120**, 403–412.

JOHNSON, C. M., STOUT, P. R., BROYER, T. C. & CARLTON, A. B. (1957). Comparative chlorine requirements of different plant species. *Plant and Soil* **8**, 337–353.

JOHNSTON, A. W. B. & BERINGER, J. E. (1975). Identification of the *Rhizobium* strains in pea root nodules using genetic markers. *Journal of General Microbiology* **87**, 343–350.

JOHNSTON, A. W. B., BEYNON, J. L., BUCHANAN-WOLLASTON, A. V., SETCHELL, S. M., HIRSCH, P. R. & BERINGER, J. E. (1978). High frequency transfer of nodulating ability between strains and species of *Rhizobium*. *Nature, London* **276**, 635–636.

JOSEY, D. P., BEYNON, J. L., JOHNSTON, A. W. B. & BERINGER, J. E. (1979). Strain identification in *Rhizobium* using intrinsic antibiotic resistance. *Journal of Applied Bacteriology* **46**, 343–350.

LEONARD, L. T. (1943). A simple assembly for use in testing of cultures of rhizobia. *Journal of Bacteriology* **45**, 523–527.

NUTI, M. P., LEPIDI, A. A., PRAKASH, R. K., SCHILPEROORT, R. A. & CANNON, F. C. (1979). Evidence for nitrogen fixation (*nif*) genes on indigenous *Rhizobium* plasmids. *Nature, London* **282**, 533–535.

PANKHURST, C. E. (1977). Symbiotic effectiveness of antibiotic-resistant mutants of fast- and slow-growing strains of *Rhizobium* nodulating *Lotus* species. *Canadian Journal of Microbiology* **23**, 1026–1033.

PATE, J. S., LAYZELL, D. B. & ATKINS, C. A. (1979). Economy of carbon and nitrogen in a nodulated and nonnodulated (NO_3-grown) legume. *Plant Physiology* **64**, 1083–1088.

SCHWINGHAMER, E. A. (1964). Association between antibiotic resistance and ineffectiveness in mutant strains of *Rhizobium* spp. *Canadian Journal of Microbiology* **10**, 221–233.

SCHWINGHAMER, E. A. (1967). Effectiveness of *Rhizobium* as modified by mutation for resistance to antibiotics. *Antonie van Leeuwenhoek* **33**, 122–136.