Improvement of Symbiotic Properties in *Rhizobium leguminosarum* by Plasmid Transfer

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Transfer of plasmid pIJ1008, a recombinant of two indigenous *Rhizobium leguminosarum* plasmids into *R. leguminosarum* strain 300 produced strain 3960, which reduced significantly more N_2 in pea root nodules than did strain 300 itself. Strain 3960 was superior to the field isolate 128C53, from which the symbiotic determinants of pIJ1008 were derived, and transfer of plasmid pIJ1008 into two other genetic backgrounds also improved symbiotic performance compared to the introduction of other nodulation plasmids.

As plasmid pIJ1008 carries genetic determinants for an uptake hydrogenase activity (Hup^+) as well as nodulation capability (Nod^+) and other determinants for symbiotic nitrogen fixation (Fix^+), the increased effectiveness of strains carrying pIJ1008 may result from their capacity to conserve energy by recovering H_2 evolved by nitrogenase.

Intact plant studies with ^{15}N showed that the superior N_2 fixation capability associated with pIJ1008 was enhanced by 2 mM-NO_3^-, a common concentration of soil N. It was also shown that, as plants grew older, the hydrogenase determined by pIJ1008 was not able to recycle all the hydrogen evolved by pea root nodules.

INTRODUCTION

The traditional method for obtaining *Rhizobium* strains with favourable symbiotic properties has been the selection of naturally occurring field isolates that produce superior growth of the host legume plant (Vincent, 1974). An alternative technique has been suggested by the demonstration that random mutagenesis of a field isolate can produce strains with enhanced rates of symbiotic N_2 fixation (Maier & Brill, 1978). A third approach might be to construct an improved *Rhizobium* strain by gene transfer so that symbiotically favourable determinants present in different isolates are combined in a single hybrid strain.

One favourable symbiotic trait is the capacity of some *Rhizobium* strains to take up H_2 (Phelps & Wilson, 1941; Dixon, 1967). *Rhizobium* strains that have the H_2-uptake system (Hup) form ATP during H_2 oxidation (Emerich *et al.*, 1979) and compensate partially for the ATP-dependent evolution of H_2 catalysed by the nitrogenase enzyme complex (Bulen & LeComte, 1966). Thus, Hup^+ *Rhizobium* strains generally are more effective symbionts than those with Hup^- phenotypes (Albrecht *et al.*, 1979).

Brewin *et al.* (1980c) recently reported that genetic determinants for hydrogenase activity (Hup^+) and nodulation capability (Nod^+) were co-transferred between *R. leguminosarum* strains during conjugation. The Hup^+ determinants apparently resided on a non-transmissible plasmid, pRL6JI, which was found in *R. leguminosarum* strain 128C53 and could be mobilized by recombination with plasmid pVW5JI (Brewin *et al.*, 1982). In the present study the pVW5JI/pRL6JI recombinant plasmid pIJ1008 was transferred into several Hup^-

R. leguminosarum strains in order to investigate whether the presence of this plasmid resulted in strains with superior symbiotic properties. Strain 128C53 has previously been shown to be a relatively efficient N_2-fixing symbiont of Alaska peas (Bethlenfalvay *et al.*, 1978) and hence, because the plasmid pIJ1008 carries many of the symbiotic determinants derived from strain 128C53 it seemed possible that the transfer of pIJ1008 to other strains of *R. leguminosarum* might lead to the genetic improvement of these strains. It would however remain an open question whether or not any such improvements were due directly to hydrogenase activity, rather than to some other unspecified symbiotic trait derived from strain 128C53.

METHODS

Rhizobium leguminosarum strains and plasmids. Strains and plasmids used are listed in Tables 1 and 2, respectively. Conditions for bacterial culture were described by Beringer (1974). Membrane crosses were described by Beringer *et al.* (1978). All transmissible plasmids used in this study carried the transposon Tn5, and the transfer of plasmids to recipient strains was monitored by the transfer of the selectable marker *kan* (kanamycin resistance), which is determined by Tn5. Validity of the constructed strains was checked by analysis of plasmids on agarose electrophoretic gels (Hirsch *et al.*, 1980). The plasmid carrying the Tn5 marker was identified by Southern transfers of plasmid gels to nitrocellulose sheets, followed by hybridization to a radioactively labelled probe DNA containing the transposon Tn5 according to the method of Hombrecher *et al.* (1981). In addition, the constructed strains were back-crossed to strain 6015 (*phe-1, trp-12 rif-392 str-37 nod-6007*) (Johnston *et al.*, 1978) and repurified transconjugant clones were tested on peas to confirm that the plasmid-borne markers *nod*[+], *fix*[+] and *hup*[+] were those expected for pVW5JI, pIJ1019 or pIJ1008. Hydrogenase activity was measured as the incorporation of $^3H_2$ into pea root nodules (Bethlenfalvay & Phillips, 1979). All strains of *R. leguminosarum* into which the plasmid pIJ1008 had been introduced formed pea root nodules that took up $^3H_2$ at approximately equal rates when assayed 25 d after inoculation (Brewin *et al.*, 1980c). The rates were similar to those for 128C53 itself (data not shown).

The strategy for the strain constructions described in Table 1 was to transfer symbiotic determinants (Nod[+] Fix[+] and sometimes Hup[+]) from two field isolates of *R. leguminosarum*, namely 128C53 and 300, into each of three different recipients, namely 300, 3622 and 16015. Because the symbiotic plasmids pRL6JI and pRL10JI resident in strains 128C53 and 300, respectively, were not self-transmissible, their effects on symbiotic performance of other strains could be tested only by recombining *in vivo* with a transmissible *R. leguminosarum* plasmid such as pVW5JI. The plasmids formed by combining pVW5JI with pRL6JI or pRL10JI, termed pIJ1008 and pIJ1019, respectively (Table 2), were transferred into strains 300, 3622 and 16015 (Table 1). The appropriate control strains 3996 (300 pVW5JI) and B118 (3622 pVW5JI) nodulated peas effectively, but strain 16015 pVW5JI is Nod[−] and Fix[−]. Thus it was necessary to construct strain 3740, a Nod[+] Fix[+] derivative of 16015 pVW5JI in which the mutant plasmid pIJ1000 was replaced by pRL10JI, the natural nodulation plasmid of strain 300 (Brewin *et al.*, 1980a, 1982).

Plant culture. Alaska pea (*Pisum sativum* L.) plants were grown in a controlled environment chamber under a 14 h/10 h light/dark cycle, 50% humidity and a photon flux density (400–700 nm) of 650 μE m^{-2} s^{-1}. The chamber temperature regime was 20/15 °C during the strain comparisons without combined N and 21/20 °C during the ontogenetic experiments with strains 3740 and 3894. The plants were grown in modified 'Leonard jar' assemblies (Leonard, 1943) with either a N-free nutrient solution (DeJong *et al.*, 1981) or the same nutrient solution supplemented to contain 2 mM-KNO_3 with 1 atom % excess ^{15}N. The nutrient solutions were adjusted to pH 7·0 and changed less than 0·5 pH units during the course of the experiments. The complete Leonard jar assemblies were autoclaved before planting. Pea seeds weighing 0·21–0·23 g and containing 7·2 ± 0·14 mg N were surface-sterilized with 95% ethanol, rinsed and germinated on sterile paper towels with distilled water. After 3 d imbibition the seedlings were selected for uniformity, planted in Leonard jar assemblies and inoculated with specific *Rhizobium* strains (Table 1).

Measurements of symbiotic associations. In the strain comparisons without combined N 13 sets of seven plants were inoculated with one of each of the 13 *R. leguminosarum* strains listed in Table 1 and were grown with N-free nutrient solution. After 35 d of growth the plants were harvested. Leaf area was determined with an area meter (LI-3000, Lambda Instruments, Lincoln, Nebr., U.S.A). Leaf dry weight and leaf N content were determined separately from those of the rest of the plant.

In the ontogenetic experiments plants were inoculated with strain 3740 (Hup[−]) or strain 3894 (Hup[+]) and grown in the presence of either N-free nutrient solution or 2 mM-KNO_3 nutrient solution. Seven plants from each treatment were harvested at six intervals starting 11 d after imbibition (day 11). At each harvest, C_2H_2-dependent C_2H_4 production (referred to less rigorously as C_2H_2 reduction) and H_2 evolution were measured on excised root systems (Bethlenfalvay & Phillips, 1977). Incubation times were 15 min for H_2 evolution and 5 min for C_2H_2

Table 1. *Characteristics of Rhizobium leguminosarum strains studied*

Rhizobium strain	Characters	Symbiotic phenotype	Reference
128C53	Field isolate	$Nod^+ Fix^+ Hup^+$	Brewin *et al.* (1980*c*)
300	Field isolate	$Nod^+ Fix^+ Hup^-$	Johnston & Beringer (1975)
3996	300 pVW5JI	$Nod^+ Fix^+ Hup^-$	This study
B121	300 pIJ1019	$Nod^+ Fix^+ Hup^-$	This study
3960	300 pIJ1008	$Nod^+ Fix^+ Hup^+$	This study
3622	Field isolate TOM	$Nod^+ Fix^+ Hup^-$	Brewin *et al.* (1980*b*)
B118	3622 pVW5JI	$Nod^+ Fix^+ Hup^-$	This study
B119	3622 pIJ1019	$Nod^+ Fix^+ Hup^-$	This study
3963	3622 pIJ1008	$Nod^+ Fix^+ Hup^+$	This study
16015	300 *str-37, spc-54*, $\Delta(nod\,fix)6007$	Nod^-	Brewin *et al.* (1980*a*)
3740	16015 pVW5JI, pRL10JI*	$Nod^+ Fix^+ Hup^-$	Brewin *et al.* (1980*a*)
3758	16015 pIJ1019	$Nod^+ Fix^+ Hup^-$	This study
3894	16015 pIJ1008	$Nod^+ Fix^+ Hup^+$	Brewin *et al.* (1980*c*)

* Introduction of pRL10JI eliminated from strain 16015 the corresponding plasmid pIJ1000 (Brewin *et al.*, 1982).

Table 2. *Characteristics of Rhizobium leguminosarum plasmids studied*

Plasmid	Description	Markers*	Approximate size (MDal)	Reference
pVW5JI	pRL4JI::Tn5	Kan-r, Tra$^+$	165	Brewin *et al.* (1980*a*)
pRL4JI	Transmissible bacteriocinogenic plasmid	Med$^+$, Tra$^+$	160	Brewin *et al.* (1980*a*)
pRL6JI	Nodulation plasmid of strain 128C53	Nod$^+$, Fix$^+$, Hup$^+$	190	Brewin *et al.* (1980*c*)
pRL10JI	Nodulation plasmid of strain 300	Nod$^+$, Fix$^+$	220	Johnston *et al.* (1981)
pIJ1000	pRL10JI $\Delta(nod, fix)$	—	195	Johnston *et al.* (1981)
pIJ1008	pVW5JI/pRL6JI recombinant	Kan-r, Tra$^+$, Nod$^+$, Fix$^+$, Hup$^+$	195	Brewin *et al.* (1982)
pIJ1019	pVW5JI/pRL10JI recombinant	Kan-r, Tra$^+$, Nod$^+$, Fix$^+$	155	Brewin *et al.* (1982)

* Kan-r indicates kanamycin resistance (60 µg ml^{-1}); Tra$^+$ means transmissible by conjugation; Med$^+$ indicates 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; Hup$^+$ indicates hydrogenase activity ($^3H_2$ incorporation).

reduction. Plant dry weights were determined after 48 h at 75 °C, and N content was measured by Kjeldahl analysis with techniques that did not detect NO_3^- or NO_2^- (Burris & Wilson, 1957). The fraction of total N derived from the NO_3^- nutrient solution was determined by mass spectrometry using the formula: fraction of plant N from $NO_3^- = $ (atom % excess ^{15}N in plant)/(atom % excess ^{15}N in solution).

Strain reisolation. At the conclusion of all experiments rhizobia were reisolated from root nodules and were identified on the basis of intrinsic drug resistance patterns (Josey *et al.*, 1979) to confirm that root nodules had been formed by the appropriate strains. Because derivatives containing pVW5JI, pIJ1008 and pIJ1019 had identical drug resistance markers, it was also necessary in some cases to run electrophoretic gels of plasmids to distinguish these derivatives (Hirsch *et al.*, 1980). Two nodules from each of four plants in each treatment were sent by air from California to Norfolk in a desiccated condition suitable for reisolation of *Rhizobium*. After surface sterilization for 15–30 s in sodium hypochlorite (10%, w/v), the nodules were rehydrated for 60 min, crushed and streaked for single colonies on plates containing complete medium (tryptone and yeast extract; Beringer, 1974). Five clones per nodule were checked for drug resistance markers and two clones per nodule for electrophoretic profiles of plasmids.

Table 3. *Effects of Rhizobium leguminosarum strains on plant growth and N assimilation*

Peas were grown for 35 d in the absence of combined N before harvest. Strain 16015 formed no root nodules.

Rhizobium strain	Plant dry weight (g)	Plant N content (mg)	Leaf area (cm²)	N concentration (%)
128C53	4·05	138	333	3·4
300	3·98	153	339	3·8
3996	4·03	140	315	3·5
B121	3·98	168	363	4·2
3960	4·58	198	386	4·6
3622	2·00	60	170	3·0
B118	1·82	57	159	3·1
B119	1·07	27	82	2·5
3963	3·75	127	306	3·4
16015	0·53	8	— *	1·5
3740	3·15	104	267	3·3
3758	2·83	114	249	4·0
3894	3·81	173	381	4·6
L.S.D.† (0·05)	0·47	16	46	0·2

* Leaves senesced before harvest.
† Least significant difference at $P \leq 0.05$, based on a one-way analysis of variance.

RESULTS

Effects of transferring various plasmids to different genetic backgrounds

Except in the special case of strain 3740, none of the introduced plasmids pVW5JI, pIJ1008 or pIJ1019 eliminated any of the resident plasmids from the recipient strains. Therefore, the derivatives of strains 300 and 3622 containing pIJ1008 carried two copies of certain *nod* and *fix* genes. It was for this reason that the pIJ1019 plasmid series was included in the study as an appropriate control for pIJ1008. Like pIJ1008, pIJ1019 is a recombinant between pVW5JI and a non-transmissible nodulation plasmid. Unlike pIJ1008, it does not carry the Hup$^+$ determinant.

When plasmid pVW5JI was transferred into strains 300 and 3622, the resulting strains 3996 and B118 maintained the capacity to fix N_2, and plant dry weight and N content associated with these strains were not significantly different from the same parameters measured for plants nodulated by the parent strains 300 and 3622 (Table 3). The transfer of pVW5JI and pRL10JI into strain 16015 produced strain 3740 which also was capable of N_2 fixation. Transferring the pVW5JI/pRL10JI recombinant plasmid pIJ1019 into strains 300 or 16015 to produce strains B121 or 3758 had little effect on symbiotic properties relative to strains 3996 and 3740, respectively, except for a slight increase in N_2 fixation. The same plasmid, pIJ1019, however, had a distinctly deleterious effect on symbiotic performance when it was transferred into strain 3622 (see strain B119, Table 3). By contrast plasmid pIJ1008, which carries *hup* determinants, produced strains with significantly better symbiotic properties than all other related strains. Strains 3960, 3963 and 3894, all of which expressed uptake hydrogenase activity in the $^3H_2$ incorporation assay, produced marked increases in plant dry weight and N_2 fixation. The plant dry weight, N content and leaf area produced by strain 3960 were also significantly greater than those of the Hup$^+$ strain 128C53, from which the *hup* determinants in pRL6JI were derived (Table 3). Total plant N concentration (Table 3) was highly correlated with total N content ($r = 0.932$, $P \leq 0.001$).

Table 4. *Symbiotic N_2 fixation in peas nodulated by Rhizobium leguminosarum strain 3740 or 3894 and grown in the absence of combined N*

Original seed N content was subtracted from the mean Kjeldahl N value of at least seven replicate plants on each harvest date.

	N_2 fixed per plant (mg)	
Plant age (d)	3740	3894
11	0	0
17	2	1
23	12	15*
30	38	66***
37	74	118***
44	117	202***

*, *** – Strain effect significant at $P \leq 0.05$ or 0.001, respectively.

Table 5. *N utilization in peas nodulated by Rhizobium leguminosarum strain 3740 or 3894 in the presence of 2 mM-NO_3^-*

Symbiotic N_2 fixation was calculated from dilution of $^{15}NO_3^-$. Original seed N content (7·7 mg) was included in total N values. Values represent the mean of at least seven replicate plants.

			N utilization (mg)					
	Total N (mg)		N_2		Solution NO_3^-		Symbiotic N_2 fixation (%)	
Plant age (d)	3740	3894	3740	3894	3740	3894	3740	3894
11	8	8	0	0	1	1	0·0	0·0
17	15	14	4	2*	4	5	26·7	14·3
23	31	38*	15	23***	10	8	48·4	60·5**
30	72	119***	45	90***	20	22	62·5	75·6***
37	162	241***	115	186***	40	48	71·0	77·2***
44	239	363***	165	279***	66	76*	69·0	76·8*

*, **, *** – Strain effect significant at $P \leq 0.05, 0.01$ or 0.001, respectively.

Effect of NO_3^- on symbiotic properties of strains 3740 and 3894

A comparison of the Hup$^-$ and Hup$^+$ strains 3740 and 3894 produced by plasmid transfer showed that strain 3894 reduced more N_2 in root nodules of plants in both the absence and presence of 2 mM-NO_3^- (Tables 4 and 5). Strain 3894 reduced significantly more N_2 than strain 3740 during the first 23 d of growth in both N treatments ($P \leq 0.05$). That advantage increased during the remaining 21 d of the study ($P \leq 0.001$) (Tables 4 and 5) and within a given N treatment plants nodulated by strain 3894 had significantly greater dry weight than those nodulated by strain 3740 for the last three harvests (Fig. 1). No significant strain effect on NO_3^- utilization was detected until the final harvest when plants nodulated by strain 3894 contained more reduced N from NO_3^- than plants nodulated by strain 3740 (Table 5). Therefore, the proportion of total reduced N that was derived from N_2 was greater over the last four harvests in plants nodulated by strain 3894 (Table 5).

In general, short-term rates of apparent N_2 fixation calculated from C_2H_2 reduction and H_2 evolution were consistent with the more definitive measures of N_2 fixation based on Kjeldahl N content and ^{15}N dilution. Plants nodulated by strain 3894 had C_2H_2-reduction rates greater than the rates of plants nodulated by strain 3740 in the same N treatment (Fig. 2). The
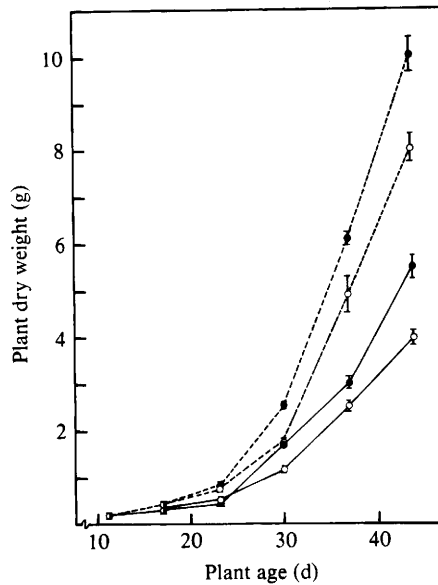
Fig. 1. Dry weight of pea plants nodulated by *Rhizobium leguminosarum* strain 3740 (○) or 3894 (●) and grown without combined N (solid line) or with 2 mM-NO_3^- (dashed line). Each point and associated vertical bars indicate the mean ± s.D. for a minimum of seven plants.

2 mM-NO_3^- treatment increased C_2H_2-reduction rates for both *Rhizobium* strains. H_2 evolution from plants nodulated by strain 3894 was lower initially than that associated with strain 3740 in both N treatments (Fig. 2). As nitrogenase activity increased on a whole plant basis in peas nodulated by strain 3894, however, H_2 evolution from that symbiotic association also increased and exceeded the H_2 evolved from the 3740 symbiosis. The relative efficiency of N_2 fixation, defined by Schubert & Evans (1976) as $1 - (H_2$ evolved/C_2H_2 reduced), was greater for Hup$^+$ strain 3894 than for Hup$^-$ strain 3740 at all points assayed (Fig. 2).

Tests for contaminating Rhizobium strains during plant growth experiments

With a few exceptions *Rhizobium* cells isolated from root nodules and tested in these experiments had characteristics identical to the original inoculant strains. A small fraction of clones (15–20%) derived from plants inoculated with strains B121 and 3740 lacked resistance to kanamycin but had the expected patterns of resistance to other drugs. Examination of the plasmid profiles of those kanamycin-sensitive derivatives of B121 and 3740 showed that they differed from the inoculant strains only by the loss of the Tn5-marked plasmids pIJ1019 and pVW5JI, respectively. In all but two of the plants where derivatives of strains B121 or 3740 were reisolated, the majority of clones were still the original B121 or 3740. The 40 clones tested for each of the other strains showed the expected patterns of drug resistance. None of the plants inoculated with the non-nodulating strain 16015 showed any evidence of root nodules or any significant increase in N content over that contained in the original seed.

DISCUSSION

This research clearly demonstrates the potential for increasing the effectiveness of the *Rhizobium*–legume symbiosis by selective transfer of plasmids. The presence of plasmid pIJ1008 in strains 3960 and 3963 was associated with 31% and 128% increases in N_2
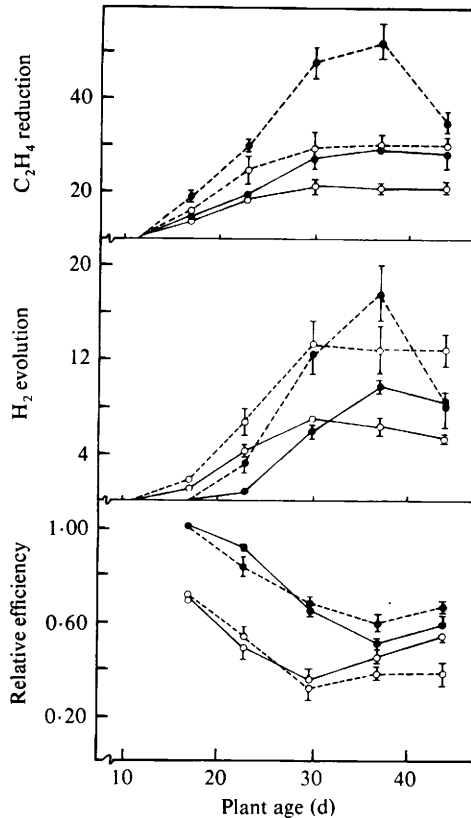
Fig. 2. Ontogenetic changes in C_2H_2 reduction, H_2 evolution, and relative efficiency of peas nodulated by *Rhizobium leguminosarum* strain 3740 (O) or 3894 (●) and grown without combined N (solid line) or with 2 mM-NO_3^- (dashed line). Each point and associated vertical bars indicate the mean ± S.D. of measurements made on a minimum of seven plants. H_2 evolution and C_2H_4 reduction were expressed as μmol per plant h^{-1}. Relative efficiency was defined as $1 - (H_2$ evolved/C_2H_2 reduced).

fixation relative to field isolates 300 and 3622 (calculated after subtracting original seed N from the values in Table 3). Furthermore, strain 3960 fixed 46 % more N_2 than field isolate 128C53, the source of pRL6JI from the Hup$^+$, Nod$^+$ and Fix$^+$ determinants of pIJ1008 were derived (Brewin *et al.*, 1980c). The simplest interpretation of the present study is that the increased effectiveness of the superior strains resulted from their common capacity to recover H_2 evolved by nitrogenase. That advantage presumably was associated with the energy conserving nature of the Hup system in *Rhizobium* bacteroids (Emerich *et al.*, 1979).

Although the simplest explanation of the results shown in Table 3 emphasizes the advantage of Hup$^+$ determinants contained in pIJ1008, more complicated explanations cannot be ignored. One such possibility is that the introduction of additional Nod$^+$ and Fix$^+$ determinants from strain 128C53 contributed to the improved performance of strains containing pIJ1008. However, the presence of the nodulation plasmid pIJ1019, which also contains additional Nod$^+$ and Fix$^+$ determinants, did not enhance the symbiotic performance of strains B121 or B119 relative to 300 or 3622, respectively. Similarly, in a previous study (DeJong *et al.*, 1981), it was shown that the presence of an additional nodulation plasmid (pRL1JI) also failed to enhance symbiotic N_2 fixation by *R. leguminosarum* strain 300. Thus, the mere presence of a second nodulation plasmid (pIJ1008) in strains 3960 and 3963 cannot in itself explain their superior symbiotic performance.

Another explanation might be that different nodulation plasmids may behave differently in various genetic backgrounds. For example, the presence of plasmid pIJ1019 had little effect on symbiotic performance in strain 300 (B121) but had a significant depressive effect in strain 3622 (B119). Similarly, strains 3740 and 3996, which appear to have identical plasmid contents, differ significantly in symbiotic proficiency. Because strain 16015 is a derivative of strain 300 (Brewin *et al.*, 1980*a*), it seems likely that in the 16015 background one or more of the chromosomal markers, for example *str-37* or *spc-54*, impaired the overall symbiotic performance of strain 3740.

The three Hup^- genetic backgrounds into which pIJ1008 was introduced can thus be graded on the basis of their symbiotic performance on Alaska peas as poor (3622), intermediate (16015) or good (300). In all cases the introduction of pIJ1008 enhanced symbiotic N_2 fixation, but the ranking order for the transconjugants remained the same as for the original strains, i.e. the strain 3622 derivative was the worst and the strain 300 derivative was the best (Table 3). It is interesting to note that the degree of enhancement by pIJ1008 was correlated inversely with the previous effectiveness of the recipient strain: i.e. 128%, 70% and 31% increases in N_2 fixation were observed for strains 3622, 16015 (i.e. strain 3740) and 300, respectively. In a previous study (DeJong *et al.*, 1981), the nodulation plasmids from three strains, which could be graded for symbiotic performance as poor (3622), intermediate (248) or good (300) were transferred into a single recipient strain (16015) of intermediate capability. The 16015 derivatives containing those nodulation plasmids (pRL5JI, pRL1JI and pRL10JI, respectively) were indistinguishable from each other in symbiotic performance, suggesting in those cases that the introduced plasmids were less important in determining symbiotic performance than was the genetic background of the host strain. By contrast, in the present study both the introduced plasmid (pIJ1008) and the genetic background of the host strain made a significant contribution to the overall level of symbiotic N_2 fixation with Alaska peas.

The identical dry weights and the similar rates of C_2H_2 reduction of plants grown on N-free solution up to day 23 (Figs 1 and 2) suggest that strains 3740 and 3894 do not differ significantly in the rate at which they form nodules on Alaska pea seedlings. The differences between the plants grown on N-free solution and those supplied with 2 mM-NO_3^- are consistent with the known importance of combined N during seedling development in this system (DeJong & Phillips, 1981). Alleviating the N stress during early seedling growth promoted symbiotic N_2 fixation and plant growth in plants nodulated with either strain. The most critical fact revealed by data in Table 5 is that the superior N_2 fixation capability associated with pIJ1008 was enhanced by the availability of 2 mM-NO_3^-, a concentration of nitrate commonly found in field soil solutions (Russell, 1973). The promotion of N_2 fixation in strain 3894 relative to 3740 was significant both on an absolute basis ($P \leq 0.001$) and on a relative basis expressed as percentage of total plant N from N_2 ($P \leq 0.05$) because strain 3894 had little effect on NO_3^- utilization until the final harvest (Table 5).

The data obtained from plants nodulated by Hup^- strain 3740 or Hup^+ strain 3894 indicate that the Hup system in these strains was not efficient enough to recover all H_2 evolved by nitrogenase. The relative efficiency values in plants nodulated with strain 3894 were near 1·0 only when root nodules were first formed and decreased to as low as 0·5 in some plants at later stages (Fig. 1). Schubert *et al.* (1977) reported that Hup^+ soybean and cowpea rhizobia maintained relative efficiencies near 1·0 for over 4 weeks. Previous research with the Hup^+ strain 128C53 also showed ontogenetic changes in the relative rates of H_2 evolution and $^3H_2$ incorporation (Bethlenfalvay & Phillips, 1979).

This work indicates that both the quantity of plant biomass produced and the N content of that biomass can be increased by selective transfer of plasmids into strains of *R. leguminosarum*. In most cases the introduced plasmids were stably maintained in the recipient strains even after one passage through pea root nodules. Because not all H_2 evolved by nitrogenase was recovered by the Hup system determined on pIJ1008, additional

opportunities exist for identifying superior Hup systems that can be transferred to *R. leguminosarum*. It will be of interest to test whether plasmid-borne *hup* determinants eventually may be used to insert Hup systems into *R. meliloti* and *R. trifolii* since no reproducible, significant $^3H_2$-incorporation activity has been measured in those organisms which nodulate the important forage crops alfalfa and clover, respectively.

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