Effect of Transfer of Symbiotic Plasmids and of Hydrogenase Genes (*hup*) on Symbiotic Efficiency of *Rhizobium leguminosarum* Strains

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Plasmid-encoded symbiotic determinants from the *Rhizobium leguminosarum* strain MA1 (817) with uptake hydrogenase activity (Hup⁺) and from the Hup⁻ strain MC1 (18a) were mobilized by recombination with the self-transmissible plasmid pVW5J1. The symbiotic determinants were transferred by conjugation from strain MA1 to strain MC1 and to a derivative of MC1 without the symbiotic plasmid, and *vice versa*, thus constructing four types of transconjugants. The determinants for a total recycling hydrogenase in strain MA1 were found to be encoded on the symbiotic plasmid.

Strain MC1 fixed 60% more N₂ in pea root nodules, determined as mg nitrogen per plant, than strain MA1. This difference was not increased in the MC1 derivative that obtained hydrogenase activity. Plants inoculated with a derivative of strain MA1, however, where the symbiotic plasmid was replaced by that of strain MC1 had a high percentage nitrogen content. It was concluded that the symbiotic plasmid and the genetic background were more important for plant nitrogen accumulation than uptake hydrogenase.

INTRODUCTION

During symbiotic nitrogen fixation where N₂ is reduced to NH₄⁺ the enzyme system responsible, nitrogenase, also reduces protons to H₂. Since the H₂ production is dependent on ATP hydrolysis (Bulen & LeComte, 1966), H₂ evolved by nitrogenase represents an energy loss. Some *Rhizobium* strains, however, possess an uptake hydrogenase (Hup) which recycles some or all of the H₂ produced by nitrogenase, whereby up to one-third of the energy lost may be regained (Schubert *et al.*, 1978). Nitrogenase can utilize ATP produced in this way as a source of energy (Emerich *et al.*, 1979).

Comparisons between plants inoculated with Hup⁺ or Hup⁻ strains have often shown that the hydrogenase-containing strains give rise to higher plant yield, expressed as mg nitrogen per plant (Schubert *et al.*, 1978; Bethlenfalvay & Phillips, 1979; Albrecht *et al.*, 1979). A *hup* point mutant of *R. japonicum* produced a lower plant yield than the Hup⁺ parent strain (Lepo *et al.*, 1981), and transfer of a plasmid with Hup determinants to Hup⁻ *R. leguminosarum* strains increased their N₂ fixation (DeJong *et al.*, 1982). With *R. leguminosarum*, however, a lack of correlation between Hup activity and plant yield has been observed (Ruiz-Argüeso *et al.*, 1978; Truelsen & Wyndaele, 1984).

Genes for nodulation and nitrogen fixation in *R. leguminosarum* seem to be located on one plasmid, the so-called symbiotic plasmid (pSym) (Krol *et al.*, 1980; Hombrecher *et al.*, 1981; Prakash *et al.*, 1981). The pSym may be self-transmissible (Hirsch, 1979; Brewin *et al.*, 1980*a*), or it can be mobilized by recombination with a transmissible plasmid (Brewin *et al.*, 1980*b*). It is then possible to study the effect of the pSym in interaction with various genetic backgrounds.

Abbreviation: pSym, symbiotic plasmid.

This study concerns the mobilization and reciprocal transfer of pSyms between two *R. leguminosarum* strains, one Hup⁺ and the other Hup⁻. The plasmid was transferred from one strain to the other, or to a derivative of the other without its own pSym, and *vice versa*. The efficiency of one pSym as opposed to a recombination of two in the two bacterial backgrounds was investigated, comparing the amounts of fixed nitrogen in pea plants. The effect of the hydrogenase system is discussed.

METHODS

Bacteria. *R. leguminosarum* strains and relevant plasmids are listed in Table 1. Strains 817 and 18a were isolated from Danish soils in our laboratory and in Research Establishment Risø, Roskilde, Denmark, respectively. Strains 2517 (carrying plasmid pVW5JI) and B151 were kindly provided by N. J. Brewin, John Innes Institute, Norwich, UK. The bacteria were grown in liquid TY medium (Beringer, 1974) or on TY agar plates with various antibiotics.

Crosses and curing of plasmids. Conjugations were done as membrane crosses as described by Beringer *et al.* (1978). The resulting transconjugants were checked for sensitivity to the donor-specific marker drug. Curing was done at 37 °C in liquid TY medium for 24 h, and single colonies from TY plates were then tested for sensitivity towards the drug to which the unwanted plasmid encoded resistance.

Plasmid analysis and DNA hybridization. Plasmids were isolated as described by Hirsch *et al.* (1980). Plasmid sizes were estimated from agarose gels (Casse *et al.*, 1979). Strains 2517 and B151 were used as references, and contained plasmids of the following sizes: 100, 165, 220, 250, 285 and 310 MDa (Brewin *et al.*, 1982). DNA homology was analysed by Southern blot hybridization according to Hombrecher *et al.* (1981). The 3.5 + 5.1 kb *EcoRI* digests of pGB25 carrying *nif* genes from *R. leguminosarum* (a generous gift from J. Hontelez, Agricultural University, Wageningen, The Netherlands), which are homologous to *nifKDH* from *Klebsiella pneumoniae*, were used as hybridization probes.

Test for the presence of pSyms. All *R. leguminosarum* strains containing pSyms produce a protein of unknown function, with a sub-unit size of 24 KDa (Dibb *et al.*, 1984). Specific antibodies against the protein (a gift from N. J. Brewin) made the corresponding gene a useful marker of pSyms in transconjugant strains. Bacteria were grown on slants for 6 d, lysed and spotted onto nitrocellulose filters. The filters were incubated with the antibodies and stained as described by Dibb *et al.* (1984). This test enabled us to determine rapidly whether a strain contained the genetic region with symbiotic functions.

Plants. Peas (*Pisum sativum* L. cv. Bodil) were sown in sterilized pots and grown with N-free nutrient solution, as described by Truelsen & Wyndaele (1984), in a growth chamber under a light intensity of 300 µE m⁻² s⁻¹.

Nitrogenase and hydrogenase activities. Enzyme activities were determined using detached nodules. Plants were harvested 4 weeks after inoculation, and the nodules from three plants in one pot were pooled. Samples of 150–200 mg fresh weight were used for each measurement. Hydrogen evolution in air and hydrogen production in 80% Ar/20% O₂ (v/v) were measured continuously with a Clark-type electrode as described by Truelsen & Wyndaele (1984). Uptake hydrogenase activity was measured amperometrically on nodules cut in half to facilitate gas diffusion through the nodule tissue according to Truelsen & Wyndaele (1984).

The amount of nitrogen fixed per plant was calculated from Kjeldahl analysis made on the plants used for nodule enzyme assays.

Reisolation of bacteria. Nodules used for enzyme assays were surface sterilized for 10 s in 95% (v/v) ethanol and then 10 min in 0.1% acidified HgCl₂, and rinsed in five changes of sterile water. The nodules were cut in half, and the plane surfaces dabbed on agar plates. The resulting colonies were tested for growth on various antibiotics and analysed for plasmid content.

RESULTS

Strains 817 (MA1) and 18a (MC1), and their plasmid donor derivatives

R. leguminosarum 817 and 18a are indigenous to Danish soils, and were chosen on the basis of their difference in Hup activity. Due to Hup activity, nodules of strain 817 do not evolve H₂ even when assayed in an atmosphere of 80% Ar/20% O₂, where total electron flow through nitrogenase is diverted to H₂ production; nodules of 18a have no Hup activity (Truelsen & Wyndaele, 1984). Strain 18a had a relative efficiency [defined as 1 – (H₂ evolution in air/H₂ evolution in Ar:O₂)] of 0.4, and 817 a total recycling capacity with a relative efficiency of 1.0. Plasmid analysis of the two strains showed that their plasmid profiles were different, and provided a useful method to distinguish them. Plasmid pR1eA2 (190 MDa) from 817 and plasmid pR1eC2 (210 MDa) from 18a carry *nif* determinants hybridizing to a *nif* probe from *R. leguminosarum*, and are referred to as pSyms.

Table 1. *R. leguminosarum* strains and relevant plasmids

Strain	Hup phenotype	Symbiotic plasmid*	Plasmid size (MDa)
MA1 and 817	Hup ⁺	pSym(MA1)=pR1eA2	190
MC1 and 18a	Hup ⁻	pSym(MC1)=pR1eC2	210
B151		pSym ⁻	
MA4	Hup ⁺	pSym(MA1)/pVW5JI=pR1eAd1	310
MC2	Hup ⁻	pSym(MC1)/pVW5JI=pR1eCd1	220
MA3		pSym(MA1) ⁻	
MC3		pSym(MC1) ⁻	
MA9 (MC2 × MA3)	Hup ⁻	pSym(MC1)/pVW5JI	220
MA11 (MC2 × MA1)	Hup ⁺	pSym(MA1)/pSym(MC1)/pVW5JI	200
MC10 (MA4 × MC1)	Hup ⁺	pSym(MC1)/pSym(MA1)/pVW5JI	190
MC13 (MA4 × MC3)	Hup ⁺	pSym(MA1)/pVW5JI	205

* Recombinant plasmids (e.g. pSym(MA1)/pVW5JI) are denoted by / between the plasmids from which they are derived. Plasmid pVW5JI was carried by strain 2517.

Drug-resistant derivatives of 817 and 18a, named MA1 and MC1 respectively, were obtained by spontaneous mutation. The symbiotic properties and plasmid profiles of MA1 and MC1 were identical to those of the wild-types, and the antibiotic markers remained stable during subculturing and nodule formation.

The self-transmissibility of the two pSyms was tested. Strain MA1 or MC1 was crossed with strain B151, which is a derivative of 128C53 without the pSym. The resulting mixture of the recipient and any recipients that had received a self-transmissible pSym from the donor was used to inoculate peas, but no nodules were formed.

Transfer functions were introduced into pSym(MA1) and pSym(MC1) by the plasmid pVW5JI carrying kanamycin and streptomycin resistance (Tn5) and regions with homology to pSyms (Brewin *et al.*, 1980b). Using the procedure described by Brewin *et al.* (1982), donor derivatives of MA1 and MC1, MA4 and MC2 respectively, were constructed, each containing a recombinant plasmid derived from the pSym and pVW5JI. The identity of the donor plasmids was confirmed by transfer to non-nodulating strains, plasmid analysis, hybridization to the *nif* probe and nodulation characteristics, which were all identical to those of the native strains.

During the strain constructions transconjugants were tested for the presence of the 24 KDa protein. It became evident that pVW5JI was incompatible with the pSyms from strains MA1 and MC1, and so some derivatives lacking pSyms were obtained. Although these strains contained pVW5JI, this plasmid was removed from the MA1 derivative by heat-curing at 37 °C, resulting in strain MA3 (non-nodulating). Attempts to cure the MC1 derivatives of pVW5JI were not successful. Nevertheless, one of the non-nodulating derivatives, MC3, was used in a subsequent cross as MC1(pSym⁻).

Transfer of pSyms between strains MA1 and MC1

The observed incompatibility between pVW5JI and the pSyms from strains MA1 and MC1 implied that introduction of a recombinant plasmid derived from the pSym and pVW5JI would either eliminate the residing pSym (or *vice versa*) or create the intended recombination with this pSym.

Four crosses were made: MC2 was donor to MA1 and to MA3, and MA4 was donor to MC1 and to MC3. Selection was made for transfer of kanamycin resistance, and in the fourth cross, plants were used as secondary selection of those recipients that had obtained nodulation ability, because Tn5 was part of both the recipient and the transconjugants. Transconjugants were obtained of the two desired categories, those containing (i) the foreign pSym and not their own, and (ii) a recombination of their own and the foreign pSym (Table 1).

The plasmid profile (Fig. 1) of transconjugant strain MA9 shows an introduced plasmid which was identified as the donor plasmid from MC2; the plasmid size was the same, the plasmid hybridized with the *nif* probe, and MA9 formed Hup⁻ nodules on peas.

Fig. 1. Plasmid profiles of *R. leguminosarum* strains. Lane *a*, MC1, lane *b*, MC3 [MC1(pSym⁻, pVW5JI⁺)], lane *c*, MA1, lane *d*, MA3 [MA1(pSym⁻, pRL7JI⁺) (pRL7JI has no symbiotic function and co-transfers with pVW5JI; Brewin *et al.*, 1982)], lane *e*, MA9 [MA3(pSym(MC1)/pVW5JI)], lane *f*, MA11 [MA1(pSym(MA1)/pSym(MC1)/pVW5JI)], lane *g*, MC10 [MC1(pSym(MC1)/pSym(MA1)/pVW5JI)], lane *h*, MC13 [MC3(pSym(MA1)/pVW5JI)]. Arrowheads indicate the bands that hybridized to the *nif* probe.

Strain MA11 (from the cross MC2 × MA1) was a deletion mutant of an original transconjugant, isolated because the large recombinant plasmid harboured by the transconjugant was unstable during nodule passage. The deleted plasmid then remained stable. The plasmid was interpreted as a recombination of pSym(MA1) and the donor pSym(MC1), since (i) nodules induced by MA11 were Hup⁺, (ii) plant size and nodule mass were significantly larger after inoculation with MA11 than with MA1 (see Table 2) and (iii) MA11 was resistant to both kanamycin and streptomycin, indicating the presence of Tn5.

Strain MC13 originated from the fourth cross and was isolated from nodules. Drug resistance and the plasmid profile (Fig. 1) showed that MC13 was a derivative of MC1, and yet the nodules had H₂-uptake activity and did not evolve H₂ in air or in 80% Ar/20% O₂. MC13 had obtained the transferable fragment (205 MDa) of the donor plasmid from MA4, and eliminated the resident pVW5JI by incompatibility.

Strain MC10 (from the cross MA4 × MC1) had lost pSym(MC1) and obtained a new 190 MDa plasmid (Fig. 1). Nodules of MC10 had a Hup activity equal to that conferred by MA1, but nodules of MC10 evolved H₂ in 80% Ar/20% O₂ at about 25% of the evolution from nodules of MC1. This was a strong indication that the 190 MDa plasmid in MC10 had resulted from a recombination between pSym(MC1) and the introduced donor pSym(MA1), during which a part of the donor plasmid was lost.

Effect of the new strains on plant yield

The two original and the four constructed strains were inoculated on pea plants. Nodule and plant parameters were measured 4 weeks after inoculation; the results are shown in Table 2. Nitrogenase activity (strains MA9 and MC1) and H₂-uptake activity were the same regardless of the genetic background in which they were expressed.

Pronounced differences resulted from comparison of parameters of plant yield. The amount of fixed nitrogen per plant was 60% higher when in symbiosis with Hup⁻ MC1 than with the Hup⁺ MA1, as a consequence of the difference in plant mass. The plants in symbiosis with the transconjugants MA11, MC10 and MC13 were also significantly larger than those with MA1,

Table 2. *Nodule and plant parameters 4 weeks after inoculation of peas with six R. leguminosarum strains*

Results are given as means of three independent cultivations $\pm$ SEM of six replicates for enzyme assays, eight replicates for weight measurements and 16 replicates for N analyses. Each replicate is a composite sample from a pot with three plants. The enzyme activities are expressed as $\mu\text{mol (g nodule fresh weight)}^{-1} \text{ h}^{-1}$. Results with the same superscript letter are not significantly different at $P \leq 0.025$.

Strain	...	MAI	MAII	MA9	MC1	MC10	MC13	Control*
H ₂ evolution in air (a)		0	0	3.8 $\pm$ 0.5	5.0 $\pm$ 2.1	0	0	—
H ₂ evolution in 80% Ar/20% O ₂ (b)		0	0	7.0 $\pm$ 3.0	8.4 $\pm$ 5.0	1.9 $\pm$ 0.6	0	—
Relative efficiency [1 - (a/b)]		1.00	1.00	0.42 $\pm$ 0.18	0.38 $\pm$ 0.12	1.00	1.00	—
H ₂ uptake		2.7 $\pm$ 0.4	2.5 $\pm$ 0.3	0	0	2.4 $\pm$ 0.9	3.5 $\pm$ 1.0	—
Plant (less nodules) dry wt (g)		0.51 $\pm$ 0.09 ^a	0.62 $\pm$ 0.07 ^b	0.54 $\pm$ 0.13 ^a	0.68 $\pm$ 0.10 ^b	0.60 $\pm$ 0.10 ^b	0.59 $\pm$ 0.09 ^b	0.37 $\pm$ 0.06 ^c
Percentage N of plants (less nodules)		4.28 $\pm$ 0.20 ^d	4.26 $\pm$ 0.11 ^d	5.03 $\pm$ 0.24 ^e	4.34 $\pm$ 0.15 ^d	4.45 $\pm$ 0.17 ^d	4.35 $\pm$ 0.16 ^d	2.88 $\pm$ 0.16 ^f
N fixed (mg N per plant)†		12.8 $\pm$ 3.5 ^e	18.1 $\pm$ 3.0 ^b	19.4 $\pm$ 6.6 ^b	20.5 $\pm$ 4.9 ^b	17.5 $\pm$ 4.8 ^b	16.7 $\pm$ 3.8 ^b	—

* Non-nodulated plants inoculated with sterile bacterial medium.

† mg N per plant (less nodules) + mg N of nodules (the percentage N, 8.77 $\pm$ 0.10, was identical for all inoculations) — mg N per control plant (10.69 $\pm$ 1.32).

meaning that their ability to accumulate nitrogen and dry matter was greater. The plants in symbiosis with MA9 had a remarkably high percentage nitrogen content. Altogether, the differences effect a division into two groups with respect to the amount of nitrogen fixed per plant, MA1 being less efficient than the rest.

DISCUSSION

The pSym of MA1 transferred into the non-nodulating strain B151 resulted in nodules with H_2 -uptake activity. The activity might theoretically be due to cooperation of the introduced genes and genes already present in B151, since the parent strain of B151 (128C53) was Hup^+ . The corresponding B151 derivative that contained the pSym of MC1, however, induced effective, Hup^- nodules, implying that the functional Hup is encoded on pSym(MA1). On the other hand, nodules of the MA1 derivative lacking its own pSym (strain MA9) did not develop Hup activity when the bacteria received nitrogenase from pSym(MC1). From this evidence it is concluded that all of the determinants needed for expression of Hup activity are carried on pSym(MA1), and are not duplicated on the rest of the MA1 genome.

The difference in H_2 evolution in 80% $Ar/20\% O_2$ between MC10 and MC13 indicated that some division exists of the Hup determinants into a structural and a regulatory part, the latter being necessary for total H_2 recycling. Christensen & Schubert (1983) have reported that one recombination event between two pSyms resulted in functional genes (Fix^+), while another event conserved structural *nif* genes but resulted in deletion of some regulatory genes.

Provided H_2 oxidation is coupled to ATP formation (Emerich *et al.*, 1979), the possession of Hup would be expected to change the electron allocation through nitrogenase in the direction of enhanced production of NH_4^+ relative to H_2 (Mortenson & Thorneley, 1979; Wassink & Haaker, 1984), thereby increasing the amount of fixed nitrogen per plant. The excess ATP could alternatively bring about larger plants, with the same result (Lepo *et al.*, 1981). Nelson & Salminen (1982), though, found coupling between H_2 oxidation and ATP formation only in some of the strains examined. Another advantage of Hup has been suggested: removal of H_2 from the nodule interior will decrease the extent of competitive inhibition of N_2 that is caused by H_2 (Hwang *et al.*, 1973). It is an open question whether H_2 diffusion through the nodule tissue is too rapid for H_2 to accumulate to inhibitory levels (Dixon, 1972), or whether H_2 concentrations normally reach levels above the nitrogenase $K_i(H_2)$ (Dixon *et al.*, 1981).

The two original strains, 817 and 18a, were chosen in the hope of improving plant productivity by recombining Hup function into an otherwise efficient strain. But neither MC13 nor MC10 seemed to benefit from the hydrogenase when the amount of fixed nitrogen per plant was compared with that of plants in symbiosis with MC1. In fact, none of the constructed strains gave higher nitrogen fixation than the parent MC1, although the measured Hup activity was high enough to prevent H_2 evolution in air. The main conclusion of this study is that both pSym(MC1) and the remainder of genetic material in MC1 have properties superior to MA1. The nitrogenase activity of pSym(MC1) may be higher than of pSym(MA1), or the nitrogenase component ratio (Fe protein/Mo-Fe protein) may be higher, favouring electron allocation to N_2 at the expense of H^+ (Mortenson & Thorneley, 1979). The influence of the genetic background may be to supply the nitrogenase system with energy and reducing power, and these traits could be more efficient in MC1. DeJong *et al.* (1981) also found that the nitrogen accumulation in pea plants was for some part dependent on the bacterial genetic background.

The interaction of pSym(MC1) and the background of MA1 in strain MA9 had a remarkable effect on the percentage nitrogen content of the plant, causing an increase of 17% (from 4.28% to 5.03%) relative to MA1. Strain MA9 could be agronomically favourable in view of the pea plant as a protein source, producing a high concentration of plant protein with low fertilizer consumption.

The lack of positive effect of Hup in strains MC10 and MC13 could be for two reasons: either strain MC1 is not adapted to utilize recycled H_2 – DeJong *et al.* (1982) suggested that a good nitrogen-fixing strain may not have the potential to become more efficient by gaining Hup – or H_2 oxidation as determined by pSym(MA1) is in fact a disadvantage. Drevon & Salsac (1984)

suggested that Hup may not be beneficial for nitrogen fixation in *R. japonicum* because oxidation of H_2 may prevent oxidation of carbohydrates. Oxidation of H_2 by Hup⁺ nodules generates less ATP than oxidation of carbohydrates using equivalent amounts of O_2 . Under O_2 -limiting conditions the presence of Hup will lower the ATP supply available for nitrogen fixation, provided carbohydrate intake is sufficient. In nodules of pea, Dixon & Blunden (1983) and Witty *et al.* (1984) have reported that nitrogenase activity was limited by O_2 and not by carbohydrates, supporting the suggestion that Hup may constitute a disadvantage. The reason for the observed increase in fixed nitrogen as a consequence of Hup⁺ plasmid transfer (DeJong *et al.*, 1982) could, according to the results from this study, be ascribed not to hydrogenase but to the accompanying genes encoded on the transferred pSym.

H_2 did not seem to build up to inhibitory levels in nodules of five of the strains listed in Table 2, since the removal of H_2 by hydrogenase gave no advantage. H_2 evolution began immediately and linearly after closure of the electrode chamber, suggesting there was no diffusion barrier. Whether the symbiosis with strain MA1 does benefit from Hup because of a more open nodule structure preventing O_2 limitation, or a restriction in the supply of carbohydrates from the plant, or the need to get rid of an unusually high nitrogenase-inhibiting H_2 production cannot be settled without a Hup⁻ isogenic mutant.

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