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Effect of Plasmid pIJ1008 from *Rhizobium leguminosarum* on Symbiotic Function of *Rhizobium meliloti*

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Plasmid pIJ1008, which carries determinants for uptake hydrogenase (Hup) activity, was transferred from *Rhizobium leguminosarum* to *Rhizobium meliloti* without impairing the capacity of the latter species to form root nodules on alfalfa. The plasmid was still present in rhizobia reisolated from the root nodules of 12 different alfalfa cultivars, but only low levels of Hup activity were detected in alfalfa.

Uptake hydrogenase (Hup) activity in symbiotic *Rhizobium* cells can oxidize H_2 produced by nitrogenase and, in some cases, couple that process to ATP production (10, 13). A recent review concluded that an efficient H_2-recycling capability is a desirable characteristic for *Rhizobium* strains (9), but no strain of *Rhizobium meliloti* that possesses significant Hup activity has been identified (15).

Genetic determinants affecting Hup activity in *Rhizobium leguminosarum* have been transferred between *R. leguminosarum* strains by conjugation (5), and a recombinant plasmid pIJ1008 (pRL4JI::Tn*5*, pRL6JI) which contains those and other symbiotic determinants (6) has been used to produce symbiotically superior strains (7). In the initial work with pIJ1008, the Alaska cultivar of *Pisum sativum* L. was used as a host plant (5, 7), but a recent report shows that two other pea cultivars, JI1205 and Feltham First, produce quite different Hup phenotypes in strains containing pIJ1008 or pRL6JI (1). Feltham First produces essentially a Hup^- phenotype with large amounts of H_2 evolved from root nodules in air, whereas JI1205 associated with the same rhizobial strains evolves less H_2 from the nodules and has at least an order of magnitude more Hup activity.

The finding that host plant cultivars can have such marked effects on the expression of the Hup phenotype in rhizobia (1) forces one to reexamine the initial report which concluded that *R. meliloti* had essentially no Hup activity (15). In that earlier study, 19 strains of *R. meliloti* were tested on the Vernal cultivar of *Medicago sativa* L., and only two strains, 102F51 and 102F65, had detectable Hup^+ phenotypes. In both cases the Hup activities measured in isolated bacteroids were less than 0.1% of those observed for Hup^+ *Rhizobium japonicum* bacteroids. Thus, one could suggest that rhizobial strains 102F51 and 102F65 contained functional Hup systems that were essentially inactive in Vernal alfalfa, just as there was almost no Hup activity determined by pIJ1008 in *R. leguminosarum* associated with Feltham First pea plants.

The current study had two objectives. First, we examined *R. meliloti* 102F65 for Hup activity in symbioses with diverse alfalfa cultivars to test whether a stronger Hup^+ phenotype than that reported in the Vernal cultivar could be observed. Second, we transferred pIJ1008 into *R. meliloti* to be certain that we were working with a strain containing Hup^+ determinants and examined the resulting strains on various alfalfa cultivars for Hup^+ phenotypes.

All bacteria used in this study were cultured under previously described conditions (2), and membrane crosses were conducted by standard methods (3). Plasmid pIJ1008 carries the transposon Tn*5*, and the transfer of this plasmid to recipient strains was monitored by the transfer of the selectable marker *kan* (kanamycin resistance), which is determined by Tn*5*. The validity of the constructed strains was checked by electrophoretic analysis of the plasmids on agarose gels (11). The plasmid carrying the Tn*5* marker was identified by Southern transfers of the plasmid gels to nitrocellulose sheets followed by hybridization to a radioactively labeled DNA probe containing Tn*5* (12). The following strains were assayed for symbiotic performance on plants: *R. meliloti* 102F65 (field isolate), B287 (field isolate), and B300 (B287[pIJ1008]); *R. leguminosarum* 128C53 (Hup^+ field isolate [4]). After analyses for symbiotic properties were completed in California, desiccated root nodules were sent to Norfolk where bacteria were reisolated and checked for the presence of pIJ1008, as described previously (7).

All plants were grown under bacteriologically controlled conditions in the absence of combined nitrogen salts. Alfalfa (*Medicago sativa* L.) cultivars tested with *R. meliloti* B287 or B300 and pea plants (*P. sativum* L. cv. JI1205) tested with *R. leguminosarum* 128C53 were grown in Leonard jar assemblies containing vermiculite (8). Alfalfa cultivars tested with *R. meliloti* 102F65 were grown on filter paper strips bathed in nutrient solution in test tubes (20 by 200 mm) covered with cotton plugs (14). Leonard jars and test tube cultures were maintained in controlled environmental chambers with 16 h of light and 8 h of darkness per day at 25 and 20°C, respectively, with 50% relative humidity. Photosynthetic photon flux density (400 to 700 nm) during the light period was 650 microeinstein $\cdot$ m^{-2} $\cdot$ s^{-1}. Hup activity was measured with the $^3H_2$ incorporation assay on segments of nodulated roots (4).

R. meliloti 102F65 formed effective root nodules on eight alfalfa cultivars as determined by the visual presence of leghemoglobin in root nodules and by plant growth relative to the growth of uninoculated plants. However, all of the symbiotic associations showed very low levels of Hup activity (Table 1). Although a few associations showed statistically significant differences in the rate of $^3H_2$ incorporation, the numerical values were several orders of magnitude less than the rate of 15 nmol of H_2 $\cdot$ mg of nodule (fresh weight)$^{-1}$ $\cdot$ h^{-1} measured in the positive control, JI1205 pea plants inoculated with *R. leguminosarum* 128C53. In contrast, the measured values were much closer to the negative

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TABLE 1. Hup activity of *R. meliloti* strains[a] tested on various alfalfa cultivars

Plant cultivar	Hup activity[b] with bacterial strain:	
	B300	102F65
4c1031	23	134
Mesilla	27	63
Vernal	34	41
Hairy Peruvian	53	94
Ranger	55	
Iroquois	72	117
Moapa	87	
Moapa 69	94	52
El Unico	97	174
Dupuits	108	
Norseman	126	19
Mesa Sirsa	140	

[a] Strain 102F65 is a soil isolate presumed to carry Hup determinants; B300 was constructed by transferring plasmid pIJ1008, which carries Hup determinants, into the soil isolate B287.

[b] Picomoles of H_2 · milligram of nodule (fresh weight)$^{-1}$ · hour^{-1}; least-significant difference (0.05): B300, 43 pmol of H_2 · mg of nodule (fresh weight)$^{-1}$ · h^{-1}: 102F65, 107 pmol of H_2 · mg of nodule (fresh weight)$^{-1}$ · h^{-1}.

control, consisting of unnodulated alfalfa root segments, which had an apparent H_2 uptake rate of 20 pmol of H_2 · mg of nodule (fresh weight)$^{-1}$ · h^{-1} which was subtracted from all values reported in Table 1. All alfalfa values in that experiment were means determined from four plants of each cultivar assayed after 5 weeks of growth.

The Hup phenotypes measured in strain B300 also were essentially Hup$^-$. A preliminary experiment in which Moapa 69 alfalfa plants were nodulated by B287 or B300 and assayed 10, 11, and 12 weeks after planting showed a higher level of $^3H_2$ incorporation activity in B300 at all ages. The mean values for the three ages were 36 ± 22 and 153 ± 73 pmol of H_2 · mg of nodule (fresh weight)$^{-1}$ · h^{-1} for B287 and B300, respectively. When B300 was inoculated onto 12 different alfalfa cultivars and assayed for $^3H_2$ incorporation activity 9 weeks after planting, there were significant differences in Hup activity among the cultivars, but the actual values were within the same low range observed for 102F65 (Table 1). The values for B300 in Table 1 are the means determined from six plants of each cultivar. The growth of inoculated plants relative to uninoculated seedlings and the visual presence of leghemoglobin in root nodules indicated that strain B300 formed effective root nodules with all 12 alfalfa cultivars

To check for the genetic stability and purity of the inoculant strains, five bacterial clones were recovered from each of 20 alfalfa nodules in each treatment. In all cases, the bacteria recovered had the expected genetic markers, i.e., those from plants inoculated with B300 were resistant to kanamycin, whereas those from plants inoculated with B287 were sensitive to kanamycin. This result indicated that pIJ1008 was stably maintained in strain B300, even after passage through alfalfa nodules. To confirm this result, two bacterial isolates from each inoculant treatment also were examined for plasmid content after lysis and agarose gel electrophoresis. As expected, ex-nodule derivatives of B300 had a plasmid of the size that is characteristic of pIJ1008, and this plasmid was absent from ex-nodule derivatives of B287.

These results confirm and extend previous work indicating that very poor expression of Hup activity is observed in *R. meliloti*. Direct examination of the Vernal-102F65 association showed that Hup activity measured by $^3H_2$ incorporation was ca. 0.3% of the positive control measured in pea root nodules formed by *R. leguminosarum* (Table 1). That value is consistent with a report with other techniques which indicated that the Vernal-102F65 association had no more than 0.1% of the Hup activity observed in Hup$^+$ soybean root nodules (15). The 10-fold variation in Hup phenotypes among alfalfa cultivars associated with 102F65 is similar to the host plant effect reported for pea plants (1). These results suggest, but do not prove, that a poorly functional Hup system is present in 102F65.

Data from strain B300 provide somewhat more definite, but still not totally convincing, evidence that *hup* determinants can be expressed in *R. meliloti*. The large difference in Hup activity on Moapa 69 between maternal strain B287 and its derivative B300 indicates that pIJ1008 had some effect on the $^3H_2$ incorporation assay. That result, in conjunction with the fact that all rhizobia that were reisolated from the nodules formed by B300 still had pIJ1008, suggests that there may have been a very low, but genuine, level of Hup activity in strain B300. Various alfalfa cultivars altered the activity over a range similar to that measured in 102F65.

The major contribution of this study is that the results show that a symbiotic plasmid, pIJ1008, from *R. leguminosarum* can be transferred to *R. meliloti* and maintained in populations of rhizobia within effective alfalfa root nodules. Whether pIJ1008, which was observed in bacteria reisolated from the nodules, was present in bacteroid cells or only in undifferentiated rhizobia is not known. The evidence indicating that pIJ1008 in B300 produced more Hup activity than in the maternal recipient B287 suggests that the plasmid may have been present and partially expressed in bacteroid cells, but this question cannot be answered conclusively until a stronger expression of the Hup phenotype, or some other marker on pIJ1008, is produced.

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LITERATURE CITED

1. **Bedmar, E. J., S. A. Edie, and D. A. Phillips.** 1983. Host plant effects on hydrogen evolution by *Rhizobium leguminosarum*. Plant Physiol. **72**:1011–1015.
2. **Beringer, J. E.** 1974. R factor transfer in *Rhizobium leguminosarum*. J. Gen. Microbiol. **84**:188–198.
3. **Beringer, J. E., S. A. Hoggan, and A. W. B. Johnston.** 1978. Linkage mapping in *Rhizobium leguminosarum* by means of R plasmid-mediated recombination. J. Gen. Microbiol. **104**:201–207.
4. **Bethlenfalvay, G. J., and D. A. Phillips.** 1979. Variation in nitrogenase and hydrogenase activity of Alaska pea root nodules. Plant Physiol. **63**:816–820.
5. **Brewin, N. J., T. M. DeJong, D. A. Phillips, and A. W. B. Johnston.** 1980. Co-transfer of determinants for hydrogenase activity and nodulation ability in *Rhizobium leguminosarum*. Nature (London) **288**:77–79.
6. **Brewin, N. J., E. A. Wood, A. W. B. Johnston, N. J. Dibb, and G. Hombrecher.** 1982. Recombinant nodulation plasmids in *Rhizobium leguminosarum*. J. Gen. Microbiol. **128**:1817–1827.
7. **DeJong, T. M., N. J. Brewin, A. W. B. Johnston, and D. A. Phillips.** 1982. Improvement of symbiotic properties in *Rhizobium leguminosarum* by plasmid transfer. J. Gen. Microbiol. **128**:1829–1838.
8. **DeJong, T. M., and D. A. Phillips.** 1981. Nitrogen stress and apparent photosynthesis in symbiotically grown *Pisum sativum* L. Plant Physiol. **68**:309–313.
9. **Eisbrenner, G., and H. J. Evans.** 1983. Aspects of hydrogen metabolism in nitrogen-fixing legumes and other plant-microbe associations. Annu. Rev. Plant Physiol. **34**:105–136.

10. **Emerich, D. W., T. Ruiz-Argüeso, T. M. Ching, and H. J. Evans.** 1979. Hydrogen-dependent nitrogenase activity and ATP formation in *Rhizobium japonicum* bacteroids. J. Bacteriol. **137**:153–160.
11. **Hirsch, P. R., A. W. B. Johnston, N. J. Brewin, M. Van Montagu, and J. Schell.** 1980. Physical identification of bacteriocinogenic, nodulation and other plasmids in strains of *Rhizobium leguminosarum*. J. Gen. Microbiol. **120**:403–412.
12. **Hombrecher, G., N. J. Brewin, and A. W. B. Johnston.** 1981. Linkage of genes for nitrogenase and nodulation ability on plasmids in *Rhizobium leguminosarum* and *R. phaseoli*. Mol. Gen. Genet. **182**:133–136.
13. **Nelson, L. M., and S. O. Salminen.** 1982. Uptake hydrogenase activity and ATP formation in *Rhizobium leguminosarum* bacteroids. J. Bacteriol. **151**:989–995.
14. **Olivares, J, J. Casadesús, and E. J. Bedmar.** 1980. Method for testing degree of infectivity of *Rhizobium meliloti* strains. Appl. Environ. Microbiol. **39**:967–970.
15. **Ruiz-Argüeso, T., R. J. Maier, and H. J. Evans.** 1979. Hydrogen evolution from alfalfa and clover nodules and hydrogen uptake by free-living *Rhizobium meliloti*. Appl. Environ. Microbiol. **37**:582–587.