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Plasmid Transfer Within and Between Serologically Distinct Strains of *Rhizobium japonicum*, Using Antibiotic Resistance Mutants and Auxotrophs†

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Methionine-requiring and pantothenic acid-requiring auxotrophs of *Rhizobium japonicum* USDA 31, as well as highly antibiotic-resistant mutants of *R. japonicum* strains USDA 31, USDA 110, USDA 138, and Webster 48, were isolated. These mutants were used to transfer the P-1 group plasmids R68.45 and RP4 within and between strains USDA 31, USDA 110, and Webster 48. Attempts to demonstrate transfer of either plasmid to strain USDA 138 were unsuccessful.

Construction of superior strains of *Rhizobium* species by genetic modification has been limited by available information on the genetics of the genus. Gene transfer by conjugation is particularly important for poorly characterized genetic systems, since relatively large portions of the genome may be transferred. In recent years considerable success has been achieved in the development of systems for plasmid-mediated gene transfer in several fast-growing *Rhizobium* species. Most such systems have used the promiscuous P-1 group plasmids R68.45 and RP4, originally obtained from *Pseudomonas aeruginosa* (1, 4). Plasmid R68.45 has been shown to transfer genetic markers between different species of fast-growing *Rhizobium* (2), and RP4 was used in developing a conjugation system for *R. meliloti* (6).

Very little is known about genetic mechanisms for gene transfer in slow-growing rhizobia. Kuykendall (5) reported the transfer of plasmids R1822 and PRD1 (a hybrid RP4) to *R. japonicum* USDA 110 and between antibiotic-resistant mutants of this same strain. However, he was unable to observe transfer of plasmid R68.45.

Data presented in this report demonstrate the transfer of both R68.45 and RP4 to *R. japonicum* and the subsequent transfer of both plasmids within and between three serologically distinct strains of *R. japonicum*.

MATERIALS AND METHODS

Bacteria. Bacterial strains described in the text are listed in Tables 1 and 2. Strain-specific fluorescent antibodies were used throughout to verify the identity and purity of each of the *R. japonicum* strains.

Media and culture conditions. Yeast extract-maltose medium contained the following constituents per liter of distilled water: 10 g of maltose; 1.0 g of yeast extract (Difco); 0.65 g of $K_2KPO_4 \cdot 3H_2O$; 0.20 g of $MgSO_4 \cdot 7H_2O$; and 0.10 g of NaCl. For yeast extract-mannitol medium, 10 g of mannitol was substituted for maltose. In minimal medium, 1.0 g of glutamate was substituted for yeast extract. All solid media contained 15 g/liter of agar, with Noble agar (Difco) used in solid minimal medium. Cycloheximide, to 200 μg/ml, was also added to solid media after autoclaving to inhibit the growth of fungal contaminants. The pH was adjusted to 7.0 with NaOH or HCl.

Most *R. japonicum* strains produce considerably less exopolysaccharide when grown on maltose as compared to mannitol (E. L. Schmidt, unpublished data). This reduces cell clumping, allowing more accurate titering and more reliable cloning of mutants. Thus, liquid cultures contained maltose, and plate matings were performed on yeast extract-maltose plates. All other operations, including titering, selection, and cloning of mutants and analysis of plate matings, were done on plates containing mannitol. Although growth in terms of cell numbers was about equal in liquid medium containing either maltose or mannitol, colonies appeared sooner and ultimately grew larger on plates containing mannitol. Plates containing mannitol also allowed greater distinction between strains based on differences in exopolysaccharide and pigment production.

Plates containing antibiotics were prepared by the addition of fresh, filter-sterilized antibiotic solution to media that was preautoclaved and cooled to about 50°C. Liquid media and plates used for the growth of bacteria containing plasmids R68.45 or RP4 were supplemented with 50 μg of kanamycin per ml.

All cultures were incubated at 30°C and were stored routinely as liquid stationary phase cultures at 4°C for 2 to 3 months without loss of viability.

To determine the wild-type level of resistance to a particular antibiotic, we streaked a strain of *R. japonicum* on a series of yeast extract-mannitol plates containing increasing concentrations of the antibiotic. The concentration of antibiotic generally ranged from 1 to 100 μg/ml. A strain was considered to be resistant at a particular concentration if it produced individual

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TABLE 1. *E. coli strains*

Designation	Genotype[a]	Source
179	*Δlac Δ(ppc-arg-ECBH) trp str rif*	Laboratory strain
1230	*pro met nal* (R68.45)[b]	A. Johnston
J53	*pro met* (RP4)[b]	E. Signer

[a] *Δlac*, Deletion in *lac* gene; *Δ(ppc-argECBH)*, deletion from *ppc* gene through *arg* genes; *met*, requires methionine for growth; *nal*, nalidixic acid resistant; *pro*, requires proline for growth; *rif*, rifampin resistant; *str*, streptomycin resistant; *trp*, requires tryptophan for growth.

[b] Plasmids R68.45 and RP4 provide resistance to ampicillin, kanamycin, and tetracycline.

TABLE 2. *R. japonicum strains*

Designation and genotype[a]	Source
R. japonicum USDA 31	USDA, Beltsville[b]
R. japonicum USDA 31 *ery-4*	This work
R. japonicum USDA 31 *kan-6*	This work
R. japonicum USDA 31 *str-7*	This work
R. japonicum USDA 31 *met-1 kan-12 str-7*	This work
R. japonicum USDA 31 *met-1 nov-7 str-7*	This work
R. japonicum USDA 31 *str-7* (RP4)	This work
R. japonicum USDA 31 *ery-4* (R68.45)	This work
R. japonicum str-7 (R68.45)	This work
R. japonicum USDA 31 *pan-1 str-7* (R68.45)	This work
R. japonicum Webster 48	Nodule isolate
R. japonicum Webster 48 *str-15*	This work
R. japonicum Webster 48 *str-15 rif-6*	This work
R. japonicum Webster 48 *str-15* (RP4)	This work
R. japonicum Webster 48 (R68.45)	This work
R. japonicum USDA 110 *rif-5*	This work
R. japonicum USDA 110 *str-7*	This work
R. japonicum USDA 110 *rif-5* (RP4)	This work
R. japonicum USDA 138	USDA, Beltsville, Md.
R. japonicum USDA 138 *str-1*	This work

[a] *ery*, Erythromycin resistant; *kan*, kanamycin resistant; *met*, requires methionine for growth; *nov*, novobiocin resistant; *pan*, requires pantothenic acid for growth; *rif*, rifampin resistant; *str*, streptomycin resistant.

[b] U.S. Department of Agriculture, Science and Education Administration, Cell Culture and Nitrogen Fixation Laboratory, Beltsville, Md.

colonies at least 0.25 mm in diameter after 7 days of incubation at 30°C.

To select antibiotic-resistant mutants, we spread 0.5 ml of stationary culture on plates containing a concentration of antibiotic 10-fold and 100-fold greater than the highest level of resistance as determined by the streak test. After 5 to 10 days at 30°C, individual resistant colonies were picked and cloned by restreaking from well-isolated colonies onto plates containing a concentration of antibiotic inhibitory to the wild-type parent. Each isolate was recloned at least twice.

The wild-type levels of resistance to antibiotics varied considerably among strains of *R. japonicum*, with USDA 31 being generally the most resistant. However, all *R. japonicum* strains tested were much more resistant than wild-type *Escherichia coli* to most antibiotics (unpublished data).

Auxotrophic mutants of *R. japonicum* USDA 31 were selected by using a modification of the carbenicillin-lysozyme enriched technique developed by Klapwijk et al. (3) for *A. tumefaciens*. Since USDA 31 was quite resistant to carbenicillin, ampicillin was used instead.

A stationary culture was washed twice, diluted 100-fold into maltose minimal media, and incubated overnight at 30°C with shaking. Ampicillin and lysozyme were then added to concentrations of 1 mg/ml and 100 μg/ml, respectively. Incubation was continued at 30°C with shaking, and an additional equal amount (as initially) of lysozyme was added every other day. After 5 days of incubation, cells were collected by passing 10 ml of culture through a sterile Nuclepore filter (0.20-μm pore size) and washed once with an additional 10 ml of maltose minimal medium. The filter was then placed on the surface of a yeast extract-maltose plate and incubated until growth was evident. The cells were suspended and washed in maltose minimal medium, and the ampicillin-lysozyme enrichment was repeated at least two more times.

After three or more enrichment cycles, cells were spread onto yeast extract-mannitol plates at dilutions to obtain ca. 100 well-spaced colonies per plate. The plates were then incubated until the colonies were 1 mm in diameter (about 5 days at 30°C) and then replicated onto mannitol-minimal plates and incubated for an additional 2 to 5 days at 30°C. Colonies which showed little or no growth on the mannitol-minimal plates were restreaked and cloned from the original yeast extract-mannitol plate. After cloning, isolates were rechecked for lack of growth on mannitol-minimal plates and then streaked onto mannitol-minimal plates supplemented with various amino acids, vitamins, or nitrogen bases to determine the required growth factor.

In initial experiments, cells were treated with the mutagen, ethyl methane sulfonate; however, since various ethyl methane sulfonate concentrations and treatment times gave no significant increase in the frequency of either rifampin or streptomycin resistance, the treatments were discontinued.

Plate matings. Crosses between *R. japonicum* strains were made by spreading 0.1 ml of stationary donor culture and 0.1 ml of stationary recipient culture together on yeast extract-maltose plates. If an antibiotic had been present in the donor culture medium, the cells were washed before spreading. After incubation at 30°C for 5 days, cells were scraped off the plate and suspended in 5 to 10 ml of yeast extract-maltose medium. Crosses between *E. coli* and *R. japonicum* were made with fivefold-fewer *E. coli* cells, and the plates were incubated for 3 days at 30°C.

For analyses, cells from the plate matings were diluted and spread on yeast extract-mannitol or minimal plates with or without the required metabolite or antibiotic or both, as appropriate. The frequency of selected markers was determined by dividing the titer

of cells from the selective plates by the titer of cells from the nonselective plates. If the frequency of selected markers in a plate mating was significantly greater than the frequency observed spontaneously in control experiments, the frequency of selected markers for the plate mating was considered to be the frequency of plasmid transfer per recipient cell.

RESULTS

The resistance characteristics of *R. japonicum* USDA 31 strains to streptomycin and kanamycin are summarized in Table 3. Streptomycin at 1 mg/ml and kanamycin at 0.6 mg/ml were the concentrations that were sufficiently selective yet still allowed good growth of the resistant strains. The antibiotic concentrations used for strains other than USDA 31 were determined by experiments similar to those reported in Table 3.

The auxotrophic mutants of *R. japonicum* USDA 31 were isolated after the third cycle of ampicillin-lysozyme selection. At this point about 50% of the colonies plated on yeast extract-mannitol plates failed to grow on minimal plates. Of 20 random colonies selected for further characterization, 1 isolate required additional methionine for growth, while the remaining 19 required pantothenic acid. It was assumed that all 19 pantothenic acid-requiring isolates were the progeny of a single parent cell.

The results of plate matings between *E. coli* carrying R68.45 or RP4 and various strains of *R. japonicum* are presented in Table 4. Lines a to d show the frequencies of kanamycin-resistant *R. japonicum* colonies in plate matings between *E. coli*(R68.45) and *R. japonicum* strains USDA 31, Webster 48, USDA 100, and USDA 138, respectively. In the crosses with *R. japonicum* strains USDA 31, Webster 48, and USDA 110, these frequencies were more than 20-fold greater than the appearance of spontaneous kanamycin resistance (compare to lines h to j, respectively). This suggests that plasmid R68.45 was transferred from *E. coli*(R68.45) to *R. japonicum* strains USDA 31, Webster 48, and USDA 110. In the cross with *R. japonicum* USDA 138, however, the frequency of kanamycin-resistant colonies was not significantly different than that observed spontaneously (cf. lines d and l). This

TABLE 3. *Resistance of R. japonicum USDA 31 to streptomycin and kanamycin*[a]

Strain	Concn of antibiotic (mg/ml)	Frequency of resistant colonies
Streptomycin		
Wild type	0.6	1.2×10^{-5}
	1.0	6.5×10^{-6}
	2.0	1.2×10^{-6}
str-7	0.6	0.88
	1.0	0.88
	2.0	0.44[b]
met-1 nov-7 str-7	0.6	1.04
	1.0	0.97
	2.0	Colonies too minute to count
Kanamycin		
Wild type	0.1	2.0×10^{-6}[b]
	0.6	3.5×10^{-8}
kan-6	0.1	0.84
	0.6	0.68
ery-4 (R68.45)	0.1	1.10
	0.6	1.05
met-1 kan-12 str-7	0.1	1.61
	0.6	1.47

[a] Cultures were diluted, spread on plates as indicated, and incubated at 30°C for 8 days.
[b] Colonies were considerably smaller than those on control plates with no antibiotic.

TABLE 4. *Plasmid transfer from E. coli to R. japonicum*

Cells plated	Selected markers	Frequency of selected markers[a]
(a) *E. coli* 1230 × *R. japonicum* USDA 31 *str-7*	Kan^r Str^r	4.2×10^{-6}
(b) *E. coli* 1230 × *R. japonicum* Webster 48	Kan^r pro^+ Met^+[b]	2.5×10^{-3}
(c) *E. coli* 1230 × *R. japonicum* USDA 110 *rif-5*	Kan^r Rif^r	1.0×10^{-5}
(d) *E. coli* 1230 × *R. japonicum* USDA 138 *str-1*	Kan^r Str^r	1.4×10^{-8}
(e) *E. coli* J53 × *R. japonicum* USDA 31 *str-7*	Kan^r Str^r	5.1×10^{-4}
(f) *E. coli* J53 × *R. japonicum* USDA 110 *rif-5*	Kan^r Rif^r	1.0×10^{-4}
(g) *E. coli* J53 × *R. japonicum* USDA 138	Kan^r Str^r Met^+ Pro^+[b]	$<4.6 \times 10^{-6}$[c]
(h) *R. japonicum* USDA 31 *str-7*	Kan^r Str^r	4.8×10^{-8}
(i) *R. japonicum* Webster 48	Kan^r Pro^+ Met^+[b]	$<5.5 \times 10^{-9}$
(j) *R. japonicum* USDA 110 *rif-5*	Kan^r Rif^r	4.3×10^{-7}
(k) *R. japonicum* USDA 138	Kan^r Str^r Met^+ Pro^+[b]	$<7.4 \times 10^{-9}$
(l) *R. japonicum* USDA 138 *str-1*	Kan^r Str^r	5.5×10^{-8}

[a] In crosses, the frequency of selected markers is determined per recipient cell (see text).
[b] Met^+ or Pro^+ or both refer to the wild-type ability to grow without the addition of methionine or proline. In these cases the cells were plated on mannitol-minimal plates plus antibiotics.
[c] *R. japonicum* USDA 138 appeared to be growth inhibited in the presence of *E. coli* J53, and final titers of USDA 138 were less than 10^6 cells per ml.

indicates that for strain USDA 138, stable plasmid transfer did not occur at a frequency greater than that of spontaneous kanamycin resistance.

Similar results are shown in plate matings with *E. coli*(RP4). Comparing line e with h and comparing line f with j suggest that RP4 was transferred to *R. japonicum* strains USDA 31 and USDA 110. However, results were again inconclusive with *R. japonicum* USDA 138 (cf. lines g and k).

To confirm that the kanamycin resistant *R. japonicum* colonies had received the R68.45 plasmid, we tested colonies listed in Table 4, line a, for nonselected resistance to ampicillin and tetracycline. (Resistance against ampicillin and tetracycline was also carried by both R68.45 and RP4.) All colonies tested showed little or no significant increase in resistance to either antibiotic. Therefore, it was necessary to transfer the plasmid back to a nonresistant *E. coli* strain to show that the kanamycin resistance was a function of plasmid R68.45.

Table 5 shows the results of plate matings between *E. coli* 179 and either *R. japonicum* USDA(R68.45) or *R. japonicum* Webster 48(R68.45). In both crosses there was a significant increase in the frequency of kanamycin-resistant colonies compared to spontaneous resistance with *E. coli* 179 alone. Three isolated colonies from each cross were then restreaked, purified, and tested for nonselected resistances also carried by R68.45. All isolates tested were resistant to ampicillin and tetracycline at levels comparable to that of the original *E. coli*(R68.45) parent (*E. coli* 1230) (Table 6).

The results of plate matings to determine whether R68.45 and RP4 could be transferred between mutants of the same *R. japonicum* strains are presented in Table 7. Transfer of R68.45 within *R. japonicum* strains USDA 31 and Webster 48 (lines a and b) occurred at very high frequencies, especially in Webster 48 in which 78% of colonies were kanamycin resistant.

TABLE 5. *Plasmid transfer from R. japonicum to E. coli*

Cells plated	Selected markers	Frequency of selected markers[a]
R. japonicum USDA 31 *str-7* (R68.45) × *E. coli* 179	Kan^r rapid growth[b]	5.0×10^{-2}
R. japonicum Webster 48(R68.45) × *E. coli* 179	Kan^r Str^r	6.7×10^{-8}
E. coli 179	Kan^r	$<4.4 \times 10^{-9}$

[a] In crosses, the frequency of selected markers is determined per recipient cell.

[b] *E. coli* colonies can be observed after overnight growth at 30°C.

RP4 also transferred within *R. japonicum* strains USDA 31, Webster 48, and USDA 110 (lines c, d, and e, respectively). In all cases the frequencies of selected colonies in the crosses were several orders of magnitude greater than in the donor or recipient controls (lines f to n).

Table 8 shows the results of plasmid transfer experiments between different strains of *R. japonicum*. The frequencies of colonies with selected markers are significantly higher than spontaneous controls in four of the five crosses, indicating plasmid transfer had occurred. These are USDA 31(R68.45) × USDA 110 (lines a, g,

TABLE 6. *Ampicillin and tetracycline resistance of E. coli recipients*

E. coli isolate	Plasmid donor	Level of resistance (μg/ml)	
		Amp	Tet
179 Kan^r no. 1	*R. japonicum* USDA 31 *str-7* (R68.45)	>100	3.0
179 Kan^r no. 2	*R. japonicum* USDA 31 *str-7* (R68.45)	>100	3.0
179 Kan^r no. 3	*R. japonicum* USDA 31 *str-7* (R68.45)	>100	3.0
179 Kan^r no. 4	*R. japonicum* Webster 48(R68.45)	>100	n/t
179 Kan^r no. 5	*R. japonicum* Webster 48(R68.45)	>100	n/t
179 Kan^r no. 6	*R. japonicum* Webster 48(R68.45)	>100	n/t
179 Control		0.3	0.3
1230 Control		>100	3.0

TABLE 7. *Plasmid transfer within the same R. japonicum strains*

	R. japonicum cells plated	Selected markers	Frequency of selected markers[a]
(a)	USDA 31 *str-7* (R68.45) × USDA 31 *ery-4*	Kan^r Ery^r	4.0×10^{-2}
(b)	Webster 48 (R68.45) × Webster 48 *str-15*	Kan^r Str^r	7.8×10^{-1}
(c)	USDA 31 *str-7* (RP4) × USDA 31 *ery-4*	Kan^r Ery^r	3.1×10^{-2}
(d)	Webster 48 *str-15* (RP4) × Webster 48 *str-15 rif-6*	Kan^r Rif^r	3.4×10^{-4}
(e)	USDA 110 *rif-5* (RP4) × USDA 110 *str-17*	Kan^r Str^r	5.2×10^{-4}
(f)	USDA 31 *str-7* (R68.45)	Kan^r Ery^r	8.3×10^{-6}
(g)	USDA 31 *str-7* (RP4)	Kan^r Ery^r	5.0×10^{-8}
(h)	USDA 31 *ery-4*	Kan^r Ery^r	2.4×10^{-7}
(i)	Webster 48(R68.45)	Kan^r Str^r	2.0×10^{-7}
(j)	Webster 48 *str-15* (RP4)	Kan^r Rif^r	3.0×10^{-8}
(k)	Webster 48 *str-15*	Kan^r Str^r	1.0×10^{-8}
(l)	Webster 48 *str-15 rif-6*	Kan^r Rif^r	6.7×10^{-8}
(m)	USDA 110 *rif-5* (RP4)	Kan^r Str^r	2.9×10^{-8}
(n)	USDA 110 *str-17*	Kan^r Str^r	7.7×10^{-9}

[a] In crosses the frequency of selected markers is determined per recipient cell. In all cases the frequency of selected markers per donor cell is significantly higher than spontaneous resistance in the donor cell control. The determination of frequency of selected markers is as described in the text.

TABLE 8. *Plasmid transfer between different R. japonicum strains*

	R. japonicum cells plated	Selected markers	Frequency of selected markers[a]
(a)	USDA 31 *pan-1 str-7* (R68.45) × USDA 110 *rif-5*	Kan^r Pan$^+$ Rif^{rb}	1.1×10^{-4}
(b)	USDA 31 *str-7* (R68.45) × USDA 138 *ery-1*	Kan^r Ery^r	4.3×10^{-7}
(c)	Webster 48(R68.45) × USDA 31 *str-7*	Kan^r Str^r	7.3×10^{-6}
(d)	Webster 48(R68.45) × USDA 110 *rif-5*	Kan^r Rif^r	5.6×10^{-6}
(e)	Webster 48 *str-15* (RP4) × USDA 110 *rif-5*	Kan^r Rif^r	3.9×10^{-6}
(f)	USDA 31 *str-7* (R68.45)	Kan^r Ery^r	5.6×10^{-7}
(g)	USDA 31 *pan-1 str-7* (R68.45)	Kan^r Pan$^+$ Rif^{rb}	$<1.4 \times 10^{-8}$
(h)	USDA 31 *str*	Kan^r Str^r	$<3.7 \times 10^{-8}$
(i)	Webster 48(R68.45)	Kan^r Str^r	9.0×10^{-8}
(j)	Webster 48(R68.45)	Kan^r Rif^r	9.7×10^{-8}
(k)	Webster 48 *str-15* (RP4)	Kan^r Rif^r	2.8×10^{-8}
(l)	USDA 110 *rif-5*	Kan^r Pan$^+$ Rif^{rb}	4.8×10^{-8}
(m)	USDA 110 *rif-5*	Kan^r Rif^r	4.3×10^{-7}

[a] In crosses the frequency of selected markers is determined per recipient cell. In all cases the frequency of selected markers per donor cell is significantly higher than spontaneous resistance in the donor cell control. The determination of frequency of selected markers is as described in the text.

[b] Pan$^+$ = growth without the addition of pantothenic acid.

and l); Webster 48(R68.45) × USDA 31 (lines c, h, and i); Webster 48(R68.45) × USDA rif 5 (lines d, j, and m); and Webster 48(RP4) × USDA 110 (lines e, k, and m).

For the cross shown in line b, USDA 31(R68.45) × USDA 138, the frequency of selected, resistant colonies was not significantly different from that for the USDA 31(R68.45) control shown in line f. This same inability to transfer R68.45 to USDA 138 was observed previously (Table 4, line d).

DISCUSSION

We have demonstrated the transfer of two conjugative plasmids, R68.45 and RP4, into three serologically distinct strains of *R. japonicum*: USDA 31, USDA 110, and Webster 48. We have also shown that either plsmid could be transferred freely within each strain and that transfer was also possible between the three strains, although at lower frequency. We were unable to demonstrate the transfer of either plasmid to *R. japonicum* strain USDA 138—either from *E. coli* or, in the case of R68.45, from *R. japonicum* 31.

A plasmid's ability to transfer itself between serologically distinct *R. japonicum* strains at relatively high frequency is an important consideration, since such plasmids would be prime candidates for conjugation systems capable of chromosomal DNA transfer. Transfer of chromosomal DNA would allow the combination of desirable qualities from different strains to form superior *R. japonicum* strains.

The inability to demonstrate plasmid transfer to USDA 138 could have several possible explanations, one being that USDA 138 may have a DNA restriction system which degrades unmodified plasmid DNA. It should also be noted that growth of USDA 138 is inhibited in the presence of *E. coli* J53 (Table 4), which may influence the transfer of RP4.

In spite of the inability to demonstrate plasmid transfer to USDA 138, it is likely that both plasmids will be easily transferable to most *R. japonicum* strains. This is based on two observations. First of all, the strains which showed plasmid transfer (USDA 31, USDA 110, and Webster 48) have some widely divergent phenotypic characteristics. For example, USDA 31 produces nonmucoid colonies on yeast extract-mannitol agar plates, shows considerable resistance to most antibiotics, and is a poor lectin binder, whereas USDA 110 and Webster 48 produce very mucoid colonies on yeast extract-mannitol plates, show significantly less antibiotic resistance than does USDA 31, and are strongly lectin binding. Secondly, we have also been able to transfer R68.45 from *R. japonicum* Webster 48(R68.45) to a cowpea *Rhizobium* isolate (unpublished data).

Attempts were made to show transfer of chromosomal antibiotic resistance markers by using R68.45 in USDA 31. However, spontaneous antibiotic resistance was too high to give an unambiguous result. Similar attempts were made by using two USDA 31 auxotrophs and selecting for the transfer of prototrophic markers from wild-type strains. These attempts also failed due to an unexplained killing phenomenon, which prevented the growth of any possible prototrophic recombinants on minimal plates.

To our knowledge the methionine- and pantothenic acid-requiring mutants used in this study are the first auxotrophs of *R. japonicum* to be reported. They should be very useful for further genetic and physiological studies of slow-growing rhizobia.

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LITERATURE CITED

1. **Beringer, J. E., and D. A. Hopwood.** 1976. Chromosomal recombination and mapping in *R. leguminosarum*. Nature (London) **264**:291-293.
2. **Johnston, A. W. B., and J. E. Beringer.** 1977. Chro-

mosomal recombination between *Rhizobium* species. Nature (London) **267**:611–613.

3. **Klapwijk, P. M., A. J. R. DeJonge, R. A. Schilperoot, and A. Rorsch.** 1975. An enrichment technique for auxotrophs of *Agrobacterium tumefaciens* using a combination of carbenicillin and lysozyme. J. Gen. Microbiol. **91**:177–182.
4. **Kondorosi, A., G. B. Kiss, T. Forrai, E. Vincze, and Z. Banâlvi.** 1977. Circular linkage map of *Rhizobium meliloti* chromosome. Nature (London) **268**:525–527.
5. **Kuykendall, L. D.** 1979. Transfer of R factors to and between genetically marked sublines of *R. japonicum*. Appl. Environ. Microbiol. **37**:862–866.
6. **Meade, H. M., and E. R. Signer.** 1977. Genetic mapping of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. U.S.A. **74**: 2076–2078.