APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1991, p. 3264–3269
0099-2240/91/113264-06$02.00/0

Vol. 57, No. 11

Transfer of the Pea Symbiotic Plasmid pJB5JI in Nonsterile Soil†

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Received 24 June 1991/Accepted 28 August 1991

Transfer of the pea (*Pisum sativum* L.) symbiotic plasmid pJB5JI between strains of rhizobia was examined in sterile and nonsterile silt loam soil. *Sinorhizobium fredii* USDA 201 and HH003 were used as plasmid donors, and symbiotic plasmid-cured *Rhizobium leguminosarum* 6015 was used as the recipient. The plasmid was carried but not expressed in *S. fredii* strains, whereas transfer of the plasmid to *R. leguminosarum* 6015 rendered the recipient capable of nodulating pea plants. Confirmation of plasmid transfer was obtained by acquisition of plasmid-encoded antibiotic resistance genes, nodulation of pea plants, and plasmid profiles. Plasmid transfer in nonsterile soil occurred at frequencies of up to 10^{-4} per recipient and appeared to be highest at soil temperatures and soil moisture levels optimal for rhizobial growth. Conjugation frequencies were usually higher in sterile soil than in nonsterile soil. In nonsterile soil, transconjugants were recovered only with strain USDA 201 as the plasmid donor. Increasing the inoculum levels of donor and recipient strains up to 10^9 cells g of soil^{-1} increased the number of transconjugants; peak plasmid transfer frequencies, however, were found at the lower inoculum level of 10^7 cells g of soil^{-1}. Plasmid transfer frequencies were raised in the presence of the pea rhizosphere or by additions of plant material. Transconjugants formed by the USDA 201(pJB5JI) × 6015 mating in soil formed effective nodules on peas.

Bacteria of the genera *Rhizobium*, *Bradyrhizobium*, and *Sinorhizobium* have received considerable attention due to their ability to form nitrogen-fixing symbioses with several legume species. The recognition that many nodulation and nitrogen fixation genes in *Rhizobium* and *Sinorhizobium* are located on high-molecular-weight symbiotic (Sym) plasmids stimulated study of the genetics of these microorganisms. Several of these Sym plasmids were demonstrated to be self-transmissible, or mobilizable by other plasmids, raising questions about the possible occurrence and extent of Sym plasmid transfer in the soil environment. Studies of the population genetics of rhizobia, using enzyme polymorphism and DNA hybridization probes, suggested that Sym plasmid transfer does occur in soil, although gene transfer appears to be a relatively rare event (16, 27). The natural existence of Sym plasmid-free rhizobia in soil is also intriguing (18) and suggests that plasmid transfer and loss are processes occurring in soil populations of rhizobia.

The construction and environmental release of genetically modified rhizobia with enhanced N_2-fixing abilities hold much promise for improving legume production systems. This potential has yet to be realized, however, because of competition for nodulation by indigenous rhizobial strains. One solution may be the breeding of legume cultivars which restrict nodulation by specific groups of rhizobia, allowing for successful inoculation with genetically improved strains (5). Before widespread release of genetically modified rhizobia into the environment occurs, information is needed about possible ecological consequences of such release. One area of concern is gene transfer, particularly plasmid transfer, to other microorganisms in soil.

To date, there has been no direct evidence of plasmid transfer among populations of rhizobia in other than highly artificial systems. In all previously published reports, in situ transfer of plasmids has been investigated in sterile soil, in artificial plant growth media such as vermiculite, or in the rhizosphere of plants grown on agar or in liquid media (4, 8, 11, 12). Since soil is a highly complex matrix with unique chemical, physical, and biological properties, virtually all of which are either destroyed or extensively altered by sterilization (26), it is critical to conduct research on gene transfer between rhizobial strains in natural soil.

In this report, we describe the transfer in natural soil of pJB5JI, a pea Sym plasmid marked with Tn*5*, from *Sinorhizobium fredii* strains to a Sym plasmid-cured *Rhizobium leguminosarum* strain. The effect of several environmental and biological factors on plasmid transfer in soil is also reported.

MATERIALS AND METHODS

Strains and growth conditions. Rhizobial strains *S. fredii* USDA 201 and HH003 and *R. leguminosarum* 6015 were obtained from B. Bohlool, NifTAL Project, University of Hawaii, Paia. All were obtained both with and without the Sym plasmid pJB5JI. The ability to nodulate peas, encoded by this plasmid, is expressed in *R. leguminosarum* strains but not in *S. fredii* (13). All strains carrying pJB5JI were resistant to kanamycin. In addition, *R. leguminosarum* 6015 was resistant to rifamycin and streptomycin. Strains were maintained at 4°C on TY medium (2), but for plasmid analysis, strains were grown in modified PA broth (13). All media were sterilized by autoclaving at 121°C for 20 min; solid media were made by the addition of 1.5% agar. Selective media contained various combinations of the following filter-sterilized antibiotics (Sigma Chemical Co., St. Louis, Mo.): kanamycin, 50 μg/ml; rifamycin, 50 μg/ml; streptomycin, 1,000 μg/ml; cyclohexamide, 100 μg/ml; and pimaricin, 50 μg/ml. Cyclohexamide and pimaricin were used together to suppress fungal growth during isolation of rhizobia from soil or nodules.

Soil. The soil used was a Waukegan Silt Loam with a pH of 5.8 in 0.01 M $CaCl_2$ and a water-holding capacity of 47%.

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† Paper no. 19167 in the Scientific Journal Series of the Minnesota Agricultural Experiment Station, St. Paul.

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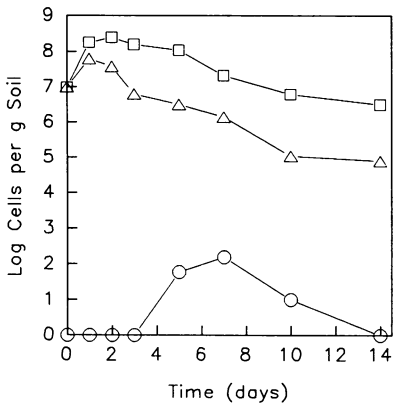

FIG. 1. Effect of incubation time on transfer of pJB5JI from *S. fredii* USDA 201 (□) to *R. leguminosarum* 6015 (△) in nonsterile soil. ○, 6015(pJB5JI) transconjugants.

This soil was free of pea-nodulating rhizobia, as determined by plant nodulation analyses. Soil was sterilized, when necessary, by autoclaving 350-g aliquots for 1 h, which was followed by 24-h incubation and a second autoclaving for 1 h.

Microcosms. Two experimental systems were used. Large microcosms consisted of a modified Leonard jar assembly (19), with the top vessel having a capacity of approximately 400 ml. The wicks were made of Whatman 3MM chromatography paper. Large microcosms were incubated in a Conviron growth chamber (Controlled Environments Ltd., Winnipeg, Manitoba, Canada) with day-night temperatures of 25 and 20°C, respectively, and a 16-h photoperiod. Large microcosms were watered from below with sterile tap water. Soil samples weighing 350 g and containing 30% moisture were planted with sterilized seeds and covered with sterile gravel. Pea seeds (*Pisum sativum*, variety Frontier) were sterilized by immersion in 95% ethanol for 5 s and then were swirled in 3% sodium hypochlorite for 3 min. The seeds were rinsed exhaustively in sterile distilled water and pregerminated in a humid environment before planting.

Small microcosms consisted of 160-ml milk dilution bottles containing moist soil equivalent to 10 g (dry weight) of soil. The soil was adjusted to 30% moisture and incubated in a humid environment at 28°C, with loose caps, unless otherwise noted. Soil moisture was maintained by weighing the bottles and adding sterile distilled water as needed.

Inoculant strains of rhizobia for both microcosms were grown in 10-g aliquots of sterile soil. Duplicate samples were assayed for rhizobial numbers by immunofluorescence, using strain-specific antibodies. Appropriate amounts of rhizobium-containing soil were used as the inoculum for nonsterile soil. For the large microcosms, 1 to 10 g of soil inoculum was used, while 1 to 5 g of soil was used for the small microcosms. Inoculum soil was well mixed with the nonsterile soil in plastic beakers, using a metal spatula.

Rhizobial isolation and enumeration. To isolate rhizobia from nodules, plants were shaken free of excess soil, and the nodules were picked, washed, and either analyzed immediately or dried in a 60°C oven. Nodules were surface sterilized (rehydrated in water if previously dried) with 3% sodium hypochlorite for 3 min and exhaustively rinsed in sterile water. Surface-sterilized nodules were crushed in 0.85% NaCl and either streaked onto plates or used to make smears on slides for fluorescent-antibody staining.

Rhizobia were separated from soil by placing 10 g of soil in a milk dilution bottle along with 95 ml of extraction fluid containing 90% 0.1 M $NH_4H_2PO_4$ and 10% hydrolyzed gelatin (10). Five drops of Tween 80 detergent and 1 drop of silicone antifoam AF72 (General Electric, Waterford, N.Y.) were also added. The bottles were shaken on a wrist-action shaker for 30 min, after which 1 ml of $CaCl_2$ (0.8 g/ml) was added and allowed to settle for 30 min. The supernatant was used for enumeration by either spread plating or immunofluorescence.

Plate counts were obtained by spread plating supernatants from soil extractions onto TY agar plates containing appropriate antibiotics and incubating at 28°C. Immunofluorescent enumeration of bacteria was done as described by Schmidt and coworkers (3, 10, 14). A total of 50 microscopic fields were counted per filter, and numbers were converted to cells per gram (dry weight) of soil. Strain-specific fluorescent antibodies were prepared by the procedures of Schmidt et al. (15).

In vitro matings and plasmid extraction. Matings were done by placing 5 loopfuls each of donor and recipient strains on a TY agar plate and mixing well to a patch

TABLE 1. Effect of inoculum levels on transfer of pJB5JI from *S. fredii* USDA 201 to *R. leguminosarum* 6015 in nonsterile soil

Inoculum level (cells g of soil^{-1})[a]	Population level (cells g of soil^{-1})[b]			Conjugation frequency
	Donor	Recipient	Transconjugant	
10^4	5.1×10^3	2.4×10^5	<10[c]	$<4.2 \times 10^{-5}$
10^5	3.3×10^6	9.6×10^5	<10	$<1.0 \times 10^{-5}$
10^6	7.2×10^6	1.3×10^6	<10	$<7.7 \times 10^{-6}$
10^7	3.4×10^7	4.8×10^6	210	4.4×10^{-5}
10^8	9.7×10^8	2.9×10^7	520	1.8×10^{-5}
10^9	8.6×10^8	2.3×10^8	2,210	9.6×10^{-6}

[a] Initial inoculum level of both donor and recipient strains.
[b] Final population levels after 7 days of incubation. Donor strain, USDA 201(pJB5JI); recipient strain, 6015.
[c] No transconjugants recovered; limit of detection, 10 cells g of soil^{-1}.

TABLE 2. Effect of temperature on transfer of pJB5JI from *S. fredii* USDA 201 to *R. leguminosarum* 6015 in nonsterile soil

Soil temp (°C)	Population level (cells g of soil^{-1})[a]			Conjugation frequency
	Donor	Recipient	Transconjugant	
15	2.1×10^7	3.4×10^6	40	1.2×10^{-5}
20	3.7×10^7	8.3×10^6	310	3.7×10^{-5}
28	4.3×10^7	6.2×10^6	280	4.5×10^{-5}
37	5.0×10^6	1.8×10^5	<10	$<5.6 \times 10^{-5}$

[a] See Table 1, footnote *b*.

TABLE 3. Effect of soil moisture on transfer of pJB5JI from *S. fredii* USDA 201 to *R. leguminosarum* 6015 in nonsterile soil

Soil moisture (%)	Population level (cells g of soil^{-1})[a]			Conjugation frequency
	Donor	Recipient	Transconjugant	
20	7.1×10^4	2.4×10^4	<10	$<4.2 \times 10^{-4}$
25	3.0×10^6	1.7×10^5	<10	$<5.9 \times 10^{-5}$
30	2.3×10^7	3.7×10^6	212	5.7×10^{-5}
35	3.8×10^7	4.9×10^6	255	5.2×10^{-5}
40	2.6×10^7	9.7×10^5	20	2.1×10^{-5}

[a] See Table 1, footnote *b*.

TABLE 4. Effect of SBM additions and different *S. fredii* donor strains on pJB5JI transfer to *R. leguminosarum* 6015 in nonsterile and sterile soils

Inoculum level (cells g of soil^{-1})	SBM (0.1%)	HH003(pJB5JI) × 6015 cross				USDA 201(pJB5JI) × 6015 cross			
		Soil population (cells g of soil^{-1})[a]			Conjugation frequency	Soil population (cells g of soil^{-1})[b]			Conjugation frequency
		Donor	Recipient	Transconjugant		Donor	Recipient	Transconjugant	
Nonsterile soil									
10^6	No	1.9×10^6	7.7×10^5	<10	$<1.3 \times 10^{-5}$	9.6×10^5	9.0×10^5	<10	$<1.1 \times 10^{-5}$
10^6	Yes	3.3×10^6	1.1×10^6	<10	$<9.1 \times 10^{-6}$	2.0×10^7	1.2×10^6	1.2×10^2	1.0×10^{-4}
10^9	No	7.6×10^8	4.3×10^8	<10	$<2.3 \times 10^{-8}$	1.3×10^9	5.2×10^8	6.3×10^2	1.2×10^{-6}
10^9	Yes	1.4×10^9	5.4×10^8	<10	$<1.9 \times 10^{-8}$	2.1×10^9	6.3×10^8	1.4×10^4	2.2×10^{-5}
Sterile soil									
10^6	No	2.8×10^6	1.1×10^6	<10	$<9.1 \times 10^{-6}$	1.1×10^7	1.5×10^6	<10	$<6.7 \times 10^{-6}$
10^6	Yes	4.8×10^6	3.4×10^6	<10	$<2.9 \times 10^{-6}$	3.5×10^7	4.4×10^6	3.0×10^2	6.8×10^{-5}
10^9	No	9.5×10^8	7.1×10^8	5.2×10^2	7.3×10^{-7}	2.4×10^9	7.3×10^8	3.2×10^4	4.4×10^{-5}
10^9	Yes	9.8×10^8	7.8×10^8	1.0×10^2	1.3×10^{-7}	2.9×10^9	1.1×10^9	2.2×10^5	2.0×10^{-4}

[a] Donor strain, HH003(pJB5JI); recipient strain, 6015.
[b] Donor strain, USDA 201(pJB5JI); recipient strain, 6015.

approximately 20 mm in size. The mating was incubated for 2 days and then washed into 10 ml of saline containing 0.01% Tween 80, mixed, diluted, and plated onto selective media for transconjugant numbers, donor and recipient background mutation frequencies, and recipient counts. All conjugation frequencies were expressed on a per-recipient basis.

Plasmid profiles for both *R. leguminosarum* and *S. fredii* strains were obtained by using the in-gel lysis procedure of Heron and Pueppke (7), with the following modifications. Strains were grown in modified PA broth (13) for 2 days, and overlay solutions contained 0.2% sodium dodecyl sulfate. Electrophoresis was carried out at 8 mA for 1 h and then at 40 mA for 3 h.

Plasmid matings in soil. In the small microcosms, conjugations were set up with *S. fredii* USDA 201(pJB5JI) and HH003(pJB5JI) as plasmid donors and *R. leguminosarum* 6015 as the potential plasmid recipient in soil. Transconjugants were recovered from the soil extract by plating on TY plates amended with rifamycin, streptomycin, and kanamycin.

A time course experiment was done with 10^6 cells g of soil^{-1}, and three replicate bottles were harvested at time intervals of 0 to 14 days. The effect of temperature was evaluated by inoculation with 10^6 cells of donor and recipient g of soil^{-1}, and then cells were incubated for 7 days at 15, 20, 28, or 37°C. The effects of soil moisture levels ranging from 20 to 40% were investigated under the same inoculation and incubation conditions just described. The effect of organic soil amendments on plasmid transfer frequency was evaluated by the addition of 1% ground dried soybean herbage (designated as soybean meal [SBM]) to soil. Other experimental factors examined included sterilization of soil and various inoculum levels from 10^4 to 10^9 cells g of soil^{-1}.

By using the same donor and recipient strains in soil contained in the large microcosms, it was possible to examine pJB5JI transfer in the rhizosphere of pea plants. The presence of pea plants also allowed the use of nodulation to directly select transconjugants from the soil microbial population. Transfer was examined in both sterile and nonsterile soil, at different inoculum levels (10^6 to 10^9 cells g of soil^{-1}), and in the presence or absence of the pea rhizosphere. Transconjugants were isolated both from within nodules and from rhizosphere soil extracts obtained at flowering of the pea plants. All of the soil in the large microcosms was considered rhizosphere soil due to extensive plant root growth. Putative transconjugants were confirmed by antibiotic resistance, fluorescent-antibody reaction, and plasmid profiles.

RESULTS

The pea Sym plasmid pJB5JI transferred from *S. fredii* HH003 and USDA 201 to *R. leguminosarum* 6015 at relatively high rates in vitro. The conjugation frequencies for patch matings were 2.7×10^{-2} and 1.7×10^{-2} with USDA 201(pJB5JI) and HH003(pJB5JI) as donors, respectively. Transfer frequencies of pJB5JI were much lower in both sterile and nonsterile soils than on solid-medium plates. The results for a time course experiment of pJB5JI transfer from strain USDA 201(pJB5JI) to 6015 in nonsterile soil are shown in Fig. 1. Population levels of both donor and recipient strains peaked after 1 to 2 days and then declined slowly, with overall higher numbers of USDA 201 than of 6015. The highest number of transconjugants was found after 7 days of incubation, although the transfer frequency was slightly higher at 10 days than at 7 days.

The effect of inoculum levels on plasmid transfer is shown in Table 1. Donor and recipient levels, as well as total transconjugant numbers, increased over the full range of inoculum levels examined, up to 10^9 cells g of soil^{-1}. However, peak transconjugant frequencies occurred at inoculum levels of 10^7 cells g of soil^{-1}. The soil environmental factors, temperature and moisture, were also examined for their effect on pJB5JI transfer in soil. As shown in Tables 2 and 3, peak transfer frequencies occurred at 28°C and 30% moisture, although higher total numbers of transconjugants occurred at 20°C and 35% moisture.

Table 4 contains data from experiments examining pJB5JI transfer in nonsterile and sterile soil, using two different *S. fredii* donor strains, two different inoculum levels, and additions of SBM. No transconjugants were isolated with HH003(pJB5JI) as the donor strain in nonsterile soil. With USDA 201(pJB5JI) as the donor in nonsterile soil, transconjugants were found only at the higher inoculum levels or with SBM additions. The highest plasmid transfer rate in nonsterile soil was 10^{-4}, with USDA 201(pJB5JI) as the donor and with SBM additions. Rhizobial population levels and plasmid transfer rates in sterile soil were higher in most cases

TABLE 5. Transfer of pJB5JI from *S. fredii* USDA 201 to *R. leguminosarum* 6015 in the pea rhizosphere

Inoculant strain	Inoculum level	Soil condition[a]	Soil population (cells g of soil^{-1})			Conjugation frequency	Nodule formation on peas
			Donor	Recipient	Transconjugant		
USDA 201(pJB5JI)	10^9	NS	8.4×10^7	<10	<10		−
6015	10^9	NS	<10	1.2×10^7	<10	$<8.3 \times 10^{-7}$	−
Consortium of USDA 201(pJB5JI) × 6015	10^6	ST	3.8×10^7	2.5×10^7	320	1.3×10^{-5}	+
	10^6	NS	1.4×10^7	6.0×10^6	40	6.7×10^{-6}	+
	10^9	ST	1.3×10^8	8.3×10^7	4,900	5.9×10^{-5}	+
	10^9	NS	7.6×10^7	2.3×10^7	1,600	7.0×10^{-5}	+

[a] NS, nonsterile; ST, sterile.

than those in nonsterile soil. Again, as in nonsterile soil, higher inoculum levels and the addition of SBM increased the transfer frequency. In sterile soil, the highest conjugation frequency was 2×10^{-4} at the 10^9 inoculum level with SBM additions.

Plasmid transfer experiments conducted in the large microcosms allowed for an examination of the effect of the pea rhizosphere on pJB5JI transfer in soil and permitted the isolation of transconjugants directly from nodules when pea nodulation had occurred. In Table 5 are listed the population levels of donors, recipients, and transconjugants in a soil consortium containing *S. fredii* USDA 201(pJB5JI) as the plasmid donor and *R. leguminosarum* 6015 as the recipient. Soil transconjugants were found at both inoculation levels in both sterile and nonsterile soil. Total numbers of transconjugants were always higher in sterile soil than in nonsterile soil. Nodules were found on the pea root systems, and putative 6015(pJB5JI) strains were isolated on the basis of kanamycin resistance and reaction with strain-specific fluorescent antibodies. Confirmation of the transfer of pJB5JI is shown in Fig. 2, in which the acquisition of a 130-MDa plasmid band (indicated by arrow) is shown in four randomly chosen pea nodule occupants. In addition, the strains of 6015 contain five other high-molecular-weight plasmids.

The effects of SBM additions and the pea rhizosphere on pJB5JI transfer in soil were compared and the results are shown in Table 6. Soil transconjugants were not recovered in the absence of either SBM or the pea rhizosphere. The pea rhizosphere had a greater effect than SBM additions on total transconjugants recovered.

DISCUSSION

Transfer of plasmids between bacterial strains in soil has been well documented in the past several years (see Stotzky for a review [20]). Within this literature, however, only a few studies have been conducted in natural soil, using microorganisms indigenous to soil, with realistic population densities and incubation conditions. While plasmid transfer may be observed and other ecological data may be obtained in artificial systems, the relevance of this information to the microbial ecology of natural environments is questionable. Only recently have gene transfer experiments in natural soil utilized indigenous soil microorganisms such as *Bacillus*, *Pseudomonas*, and *Streptomyces* species (23–25).

To date, all published reports examining gene transfer among rhizobial strains have been conducted in sterile systems (4, 8, 11, 12). Transfer of the pea Sym plasmid pJB5JI to a strain of *R. meliloti* in a sterile sand-vermiculite-charcoal mixture has been reported (4). More recently, the transfer of a symbiotic megaplasmid between rhizobia strains in the rhizosphere of alfalfa plants grown on sterile agar slants has also been demonstrated (11). Results of the current study constitute the first example of transfer of a symbiotic plasmid between *Rhizobium* strains in soil. The plasmid used, pJB5JI, was shown to transfer in nonsterile soil from *S. fredii* USDA 201 to *R. leguminosarum* 6015, and the resulting transconjugants were capable of forming nodules on pea plants. The number of transconjugants recovered was always higher in sterile soil than in nonsterile soil. In some cases, transconjugants were found only in sterile soil.

In general, soil conditions optimal for in vitro bacterial growth have also proven optimal for plasmid transfer in soil (20). This relationship was corroborated in the current study, with peak conjugation frequencies of pJB5JI between *S. fredii* and *R. leguminosarum* strains found at 28°C and 30 to 35% soil moisture. In a report examining plasmid transfer in sterile soil among *Bacillus* strains (21), only low numbers of transconjugants were recovered at 15°C, whereas much

TABLE 6. Effect of the rhizosphere and SBM additions on pJB5JI transfer from *S. fredii* USDA 201 to *R. leguminosarum* 6015 in nonsterile soil

Inoculant strain	Inoculum level	Soil condition[a]	Soil population (cells g of soil^{-1})[b]			Conjugation frequency
			Donor	Recipient	Transconjugant	
USDA 201(pJB5JI)	10^6	Plant	2.6×10^7	<10	<10	
6015	10^6	Plant	<10	9.3×10^6	<10	$<1.1 \times 10^{-6}$
Consortium of USDA 201(pJB5JI) × 6015	10^6	Plant	3.0×10^7	1.9×10^7	1,630	8.6×10^{-5}
	10^6	No plant	1.8×10^6	2.3×10^6	<10	$<4.3 \times 10^{-6}$
	10^6	No plant, SBM	8.9×10^6	4.6×10^6	170	3.7×10^{-5}

[a] Plant, presence of pea plant; SBM, addition of 0.1%.
[b] Donor strain, USDA 201(pJB5JI); recipient strain, 6015.

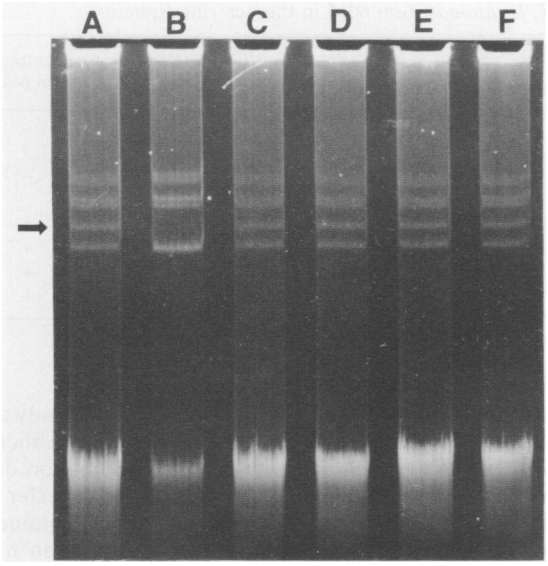

FIG. 2. Plasmid profiles of putative pJB5JI transconjugants from *S. fredii* USDA 201(pJB5JI) × *R. leguminosarum* 6015 mating in nonsterile soil. Lanes A and B, control strains of 6015(pJB5JI) and 6015. Lanes C through F, transconjugants isolated from nodules. The arrow indicates the position of the 130-MDa pJB5JI.

higher numbers of transconjugants were found at 27°C, at 2 days of incubation. Detailed studies of the effect of temperature on plasmid transfer in soil have not been reported as yet, but of interest is a study examining in situ plasmid transfer among aquatic bacteria (1). River water temperatures of between 6 and 21°C were found to have a positive linear relationship with the $\log_{10}$ plasmid transfer frequency.

Transfer of bacterial plasmids by conjugation requires actual cell-to-cell contact; the spatial separation of bacteria in the high-surface-area matrix of soil is likely to minimize this contact (20). In liquid systems, plasmid transfer kinetics have been fitted to a simple mass-action model (9). There has been little research examining the kinetics of plasmid transfer on solid surfaces, although a recent report has suggested that, because of a lack of mixing, plasmid transfer on solid surfaces is much more dependent on initial inoculum rates than are liquid matings (17). In the current research, higher inoculum levels increased the number of donors, recipients, and transconjugants recovered. At the low inoculum levels, the bacterial populations may have been too low and well dispersed in soil, making the chances of bacterial strains encountering each other for conjugation small.

The lower transfer frequencies of pJB5JI at higher inoculum levels (Table 6) could possibly be explained by a more rapid decline of rhizobial numbers at the higher inoculum levels and hence a reduced activity level of these rhizobia. Inoculation of rhizobia into natural soils at high numbers usually results in a rapid decline in viable cells, amounting to several logs, and then stabilization of rhizobial numbers at some carrying point for that particular soil (6). If rhizobial numbers were sharply decreasing, as occurred in the current study at the higher inoculum levels during 7 days of incubation, surviving cells may not have been in a physiological state receptive to conjugation. Thus, it is important to use realistic inoculum levels in microbial ecology studies involving microcosms.

If, as it appears, gene transfer frequencies in soil are dependent on microbial numbers and activity, two important niches of enhanced genetic exchange in soil may be the rhizosphere and microsites associated with fresh organic residues. Since soil at large is essentially oligotrophic and bacteria may be greatly dispersed over its high surface area, these two niches may be important as sites of increased gene transfer activity among soil microorganisms. Reports have shown the presence of the plant rhizosphere to increase transconjugant number and the transfer frequency of RP4 between *Pseudomonas* strains compared with plasmid transfer in bulk soil (22–24). Also reported in these studies was that organic matter additions (dried wheat plant tissue) did not stimulate plasmid transfer, indicating that crop residues do not mimic the rhizosphere effect on plasmid transfer. In the current study, however, SBM additions did have a positive effect on plasmid transfer, but it was not equal to the effect of the pea rhizosphere.

In summary, transfer of the pea symbiotic plasmid pJB5JI was detected among rhizobial strains present as members of consortia inoculated into natural soil. Plasmid transfer was demonstrated in soil that was neither sterilized nor amended with organic nutrients. Optimal plasmid transfer frequencies appeared to occur at soil temperatures and moisture contents ideal for growth of the rhizobial strains. Plasmid transfer frequencies were increased by the presence of the pea rhizosphere and by SBM additions, supporting the hypothesis that the rhizosphere and crop residues may be active sites for heterotrophic microbial gene transfer events in soil.

ACKNOWLEDGMENTS

We thank B. Bohlool for bacterial strains, M. Sadowsky for help in plasmid isolations, and C. Rosen for pea seeds.

This work was supported in part by grant BSR-8604947 from the National Science Foundation. A University of Minnesota Graduate School Dissertation Fellowship to B. Kinkle is also gratefully acknowledged.

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