Journal of General Microbiology (1974), **84**, 188–198
Printed in Great Britain

R Factor Transfer in *Rhizobium leguminosarum*

By J. E. BERINGER
Department of Genetics, John Innes Institute, Colney Lane, Norwich NOR 70F

(*Received* 19 *April* 1974)

SUMMARY

R factors of the compatibility class P were transferred between strains of *Escherichia coli* K12 and *Rhizobium leguminosarum*. These R factors were stable in *R. leguminosarum* and conferred similar levels of antibiotic resistance to those in the corresponding R^+ *E. coli* K12 hosts, with the exception of carbenicillin resistance which was greatly reduced. Transfer between *R. leguminosarum* strains was by conjugation and was stimulated by conditions favouring spheroplast formation. R factor mediated recombination could not be demonstrated.

INTRODUCTION

Rhizobium leguminosarum is potentially a particularly suitable species for use in genetical studies of symbiotic nitrogen fixation as it is the partner of a legume, the pea (*Pisum*), that is itself well-known genetically. However, though there have been reports of transformation (Balassa, 1963; Raina & Modi, 1972), transduction (Kowalski, 1971; Sik & Orosz, 1971) and conjugation (Heumann, 1968; Heumann, Pühler & Wagner, 1971, 1973) in various species of Rhizobium, no suitable method of genetic analysis exists for *R. leguminosarum*.

The R factor transfer experiments described in this paper were initiated as a method for determining the ability of these bacteria to transfer DNA by conjugation and in the hope that R factor mediated recombination could be demonstrated. R factors belonging to the compatibility class P were chosen, as members of this class have been shown to be transferable between different genera of bacteria (Sykes & Richmond, 1970; Roe, Jones & Lowbury, 1971; Datta *et al.* 1971; Datta & Hedges, 1972*a*; Olsen & Shipley, 1973) and more specifically because transfer of such R factors was reported to occur from *Escherichia coli* to *Rhizobium trifolii* and *R. meliloti* (Datta *et al.* 1971; Datta & Hedges, 1972*a*) and also between *R. lupini* strains (Pühler, Burkardt & Heumann, 1972).

METHODS

Bacterial strains and plasmids are listed in Table 1.

Isolation of R. leguminosarum strains. Pea root nodules were cut from the root system, still attached to a small piece of root to eliminate damage, washed in water and then treated with sodium hypochlorite (12%, w/v, available chlorine) for 3 to 10 min, depending on their size. The nodules were then washed twice in sterile distilled water, broken open with a sterile needle and the contents streaked out on TY agar plates (defined below). After 5 to 7 days' incubation, Rhizobium-like colonies were subcultured to further TY plates for single colony isolation. Initial characterization of strains was by their ability to grow on a number of different complete and minimal media; the final characterization was by a nodulation test.

Table 1. *Bacteria and plasmids*

Bacteria	Markers	Reference or source
Rhizobium leguminosarum strains*		
240, 262, 301	Wild-types	Isolated from pea root nodules
41	*ade ura str-r*	NTG mutagenesis of 301
107	*ilv*	NA mutagenesis of 240
137	*ilv his str-r*	NTG mutagenesis of 107
145	*leu*	NA mutagenesis of 301
147	*leu*	NA mutagenesis of 262
196	*pur*	NA mutagenesis of 301
209	*trp*	NA mutagenesis of 301
Escherichia coli K12 strains		
J5-3*nal-r*	F⁻ *pro met nal-r*	Clowes & Hayes (1968) Dr E. Meynell
RC24	F⁻ *thr leu thi lac str-r*	Lawn & Meynell (1970) Dr E. Meynell
J6-2	F⁻ *pro trp his lac*	Clowes & Hayes (1968) Dr N. Datta

R factors	Resistance markers†	Compatibility group	Source	References
RP4	A,T,K	P	Dr N. Datta	Holloway & Richmond (1973)
R6886	A,T,K	P	Dr E. J. L. Lowbury	Holloway & Richmond (1973)
RK2	A,T,K	P	Dr L. Ingram	—
R751	Tp	P	Dr N. Datta	Jobanputra & Datta (1974)
R702	S,T,K,Su,Hg	P	Dr N. Datta	—
R40a	K,Su,A	C	Dr N. Datta	—
R57b	Ge,A,C,Su	C	Dr N. Datta	Datta & Hedges (1972*b*)
R144*drd*3	K	I	Dr E. Meynell	Cooke, Meynell & Lawn (1970)
R1*drd*19	K,C,A,S,Su	F	Dr E. Meynell	Cooke *et al.* (1970)

* The *R. leguminosarum* strains were tested for their symbiotic properties as described in Methods: 301, 240, 262, 196, 209 and 209(RP4) were infective and effective; 145 and 147 were infective and ineffective; 41 was non-infective.

† Symbols for resistance to: ampicillin (A), tetracycline (T), kanamycin/neomycin (K), streptomycin (S), sulphonamides (Su), mercury (Hg), gentamycin (Ge), chloramphenicol (C), trimethoprim (Tp).

Media. All media were made with distilled water and, when required, were solidified with 1·5% Oxoid No. 3 agar. NB and NA were Oxoid nutrient broth and agar. MM was the minimal medium of Vogel & Bonner (1956). TY contained, per litre, 5 g Difco Bacto-Tryptone, 3 g Difco Bacto-yeast extract and 1·3 g $CaCl_2.6H_2O$. SY was the minimal medium of Sherwood (1970) modified by using sodium succinate at 1·35 g/l in place of mannitol; after autoclaving, biotin, thiamine hydrochloride and calcium pantothenate were added, each to a final concentration of 1 mg/l. VS was Vincent's (1970) yeast sucrose (phage) broth.

Antibiotics were used as freshly prepared solutions in distilled water and were added to media at the following final concentrations (mg/l). For *E. coli*: kanamycin sulphate, 25; trimethoprim, 10; carbenicillin, 100 for selection of R factor transfer and 1000 to demonstrate high level resistance; tetracycline HCl, 10. For *R. leguminosarum*: kanamycin sulphate, 25; trimethoprim, 50; carbenicillin, 50; streptomycin sulphate, 500; tetracycline HCl, 10. The antibiotic resistance patterns of strains were tested by means of Oxoid Multodiscs and by growth on antibiotic-supplemented media. Trimethoprim lactate was a gift from

J. S. Inman of The Wellcome Foundation Ltd, Berkhamsted, Hertfordshire. Other antibiotics were purchased as proprietary brands.

R factor transfer. For *E. coli*, late log-phase nutrient broth cultures (about 5×10^8/ml) were mixed in a donor–recipient ratio of about 1:5 and added to an equal volume of fresh pre-warmed NB. Crosses were incubated without shaking at 37 °C for 1 h, diluted in the buffer of Clowes & Hayes (1968) and plated on selective media.

For *R. leguminosarum*, late log-phase cultures (about 5×10^9/ml) were prepared by growing donors and recipients separately on TY slants at 28 °C for 48 h and washing the bacteria off the slants with VS medium. Equal (1 ml) volumes of donors and recipients were added to 8 ml VS and incubated with gentle shaking for 20 h unless otherwise indicated. Samples were diluted in distilled water and plated on selective minimal media. Frequencies of transfer were calculated per recipient.

In crosses between *E. coli* and *R. leguminosarum* and where more than one recipient was used, donors and recipients were cultured as above. Equal (1 ml) volumes of donor and recipient(s) were then added to VS medium to a final volume of 10 ml unless otherwise indicated. Mating was for 20 h at 28 °C with gentle shaking and samples were diluted in buffer before plating on selective minimal media. Frequencies of transfer were calculated per recipient.

Plate crosses were performed by replica plating plates carrying patches of up to six strains of *R. leguminosarum* on to plates spread with a dense suspension of bacteria of the R^+ parent. The resulting plate crosses were incubated at 28 °C for 24 h and then replicated to suitable selective media. This procedure was based on that used with *Streptomyces coelicolor* by Sermonti & Casciano (1963) and by Hopwood, Harold, Vivian & Ferguson (1969).

R^+ transconjugants derived from all crosses were routinely tested for non-selected markers of either parent and for the acquisition of all R factor mediated resistances. Control platings of donors and recipients were made to determine the spontaneous mutation rate of markers selected in crosses. In *R. leguminosarum* spontaneous resistance to tetracycline was never observed and that to kanamycin was infrequent (about 10^{-9}/cell plated) and was readily lost. For crosses in liquid medium, viable counts of donors, recipients and R^+ transconjugants were taken from the average of three plate counts at suitable dilutions on selective media.

Mutagenesis. Auxotrophic mutants of *R. leguminosarum* were induced by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) using the following procedure, based on that of Scherrer (1970). Log-phase cultures growing in VS (pH about 7·2) at 28 °C were treated with 200 mg NTG/l for 1 h; the bacteria were washed twice in distilled water and then resuspended in VS and incubated for about 20 h to allow segregation and expression of mutations. Suitable dilutions were plated on TY and colonies growing on these plates were replicated to SY medium for the detection of auxotrophic mutants. SY medium was supplemented with sodium succinate to reduce the amount of exopolysaccharide which is produced by rhizobia growing on minimal media containing glucose or mannitol (Dudman, 1964). This greatly increased the number of individual colonies that could be handled per plate (200 to 300 as opposed to 10 to 20), because the final size of colonies on succinate medium was governed more by the volume of bacteria present than by the amount of exopolysaccharide that they could produce. Multiply-marked mutants were produced by repeated NTG treatment. Nitrous acid (NA) mutagenesis was as described by Kaudewitz (1959).

Spontaneous streptomycin-resistant mutants were obtained by plating about 10^8 bacteria on TY supplemented with 1 mg streptomycin/ml.

Table 2. *R factor transfer between E. coli and R. leguminosarum*

Crosses were performed as described in Methods using a mixed *E. coli–R. leguminosarum* recipient culture in 40 % NB+60 % VS as the mating medium.

Cross		Frequency of transfer of R factor (per recipient)	
Donor	Recipients	To *E. coli*	To *R. leguminosarum*
E. coli J5-3*nal-r*(RP4)	*R. leguminosarum* 41 *E. coli* RC24	$4{\cdot}9\times10^{-3}$	$6{\cdot}2\times10^{-5}$
E. coli J5-3*nal-r*(R6886)	*R. leguminosarum* 41 *E. coli* RC24	$3{\cdot}3\times10^{-4}$	About $1{\cdot}0\times10^{-7}$
E. coli J5-3*nal-r*(RK2)	*R. leguminosarum* 41 *E. coli* RC24	$2{\cdot}2\times10^{-3}$	$5{\cdot}7\times10^{-5}$
R. leguminosarum 145(RP4)	*R. leguminosarum* 41 *E. coli* J5-3*nal-r*	$2{\cdot}8\times10^{-4}$	$2{\cdot}2\times10^{-3}$
R. leguminosarum 145(R6886)	*R. leguminosarum* 41 *E. coli* J5-3*nal-r*	$6{\cdot}8\times10^{-5}$	$7{\cdot}4\times10^{-6}$
R. leguminosarum 145(RK2)	*R. leguminosarum* 41 *E. coli* J5-3*nal-r*	$6{\cdot}9\times10^{-4}$	$1{\cdot}5\times10^{-3}$
E. coli J6-2(R751)	*R. leguminosarum* 41 *E. coli* J5-3*nal-r*	$2{\cdot}3\times10^{-3}$	$2{\cdot}0\times10^{-5}$
E. coli J6-2(R702)	*R. leguminosarum* 137 *E. coli* J5-3*nal-r*	$2{\cdot}7\times10^{-2}$	$3{\cdot}1\times10^{-4}$

Nodulation test. Peas (*Pisum sativum*), var. Wisconsin Perfection, were washed in 75 % ethanol and placed in distilled water for 10 min. The water was replaced by sodium hypochlorite (12 %, w/v, available chlorine) and left for 30 min. The peas were then rinsed in sterile distilled water and transferred to Petri dishes containing TY agar and incubated for 5 days at about 22 °C in the dark. Sterile germinated seeds were then transferred to 250 ml conical flasks containing about 100 ml of sterile moistened vermiculite at a pH of about 7. The flasks were incubated in the dark, at about 22 °C, until the etiolated plumules reached the cotton wool plugs. The plumules were pulled past the plugs and about 200 ml of sterile nitrogen-free mineral salts solution (Cartwright, 1967) were added. This medium contained the *R. leguminosarum* strain to be tested at about 10^6 bacteria/ml. Treated flasks were then covered to keep light from the plant roots and were transferred to a glass-house. Nodules were usually visible 3 to 4 weeks after infective bacteria had been added, and significant differences in foliage related to the effectiveness of nodulation were apparent about 6 weeks after inoculation. In tests where genetically marked bacteria were used, bacteria were re-isolated from nodules and tested for their phenotype by plating on suitable selective media.

RESULTS

Transfer of R factors between E. coli and R. leguminosarum

The P group R factors used in this study were found to be transferable from *E. coli* K12 to *R. leguminosarum* and between *R. leguminosarum* strains in liquid medium crosses (Table 2). All the antibiotic resistance markers carried by these R factors were expressed in *R. leguminosarum* and, with the exception of carbenicillin resistance, levels of resistance were comparable with those of the R^+ *E. coli* donor. Expression of carbenicillin resistance in R^+ *R. leguminosarum* strains was poor (about 50 μg/ml as opposed to 'wild-type' resistance of 20 to 25 μg/ml), but when the R factors were transferred back to *E. coli* full expression

Table 3. *Effect of NB or glycine on R factor transfer*

Crosses were performed as described in Methods except that NB or glycine were added to the mating medium at the concentration shown. Selection for R factor transfer was by kanamycin in suitably supplemented minimal media.

Cross	Viable count per ml			R^+ recipients per recipient	NB in medium (%)
	R^+ donors	Recipients	R^+ recipients		
41(RP4) × 145	$4{\cdot}9 \times 10^7$	$1{\cdot}7 \times 10^9$	$\sim 2 \times 10^2$	$\sim 1{\cdot}2 \times 10^{-7}$	0
	$3{\cdot}5 \times 10^8$	$3{\cdot}1 \times 10^9$	$1{\cdot}1 \times 10^4$	$3{\cdot}6 \times 10^{-6}$	10
	$2{\cdot}9 \times 10^8$	$2{\cdot}4 \times 10^9$	$1{\cdot}5 \times 10^4$	$6{\cdot}3 \times 10^{-6}$	20
	$6{\cdot}2 \times 10^8$	$1{\cdot}7 \times 10^9$	$4{\cdot}4 \times 10^3$	$2{\cdot}6 \times 10^{-6}$	30
	$8{\cdot}1 \times 10^7$	$1{\cdot}3 \times 10^8$	$8{\cdot}1 \times 10^3$	$6{\cdot}2 \times 10^{-5}$	40
	$\sim 1 \times 10^6$	$\sim 3 \times 10^6$	$1{\cdot}0 \times 10^3$	$\sim 3{\cdot}3 \times 10^{-4}$	50*
	$\sim 6 \times 10^5$	$\sim 2 \times 10^6$	$< 1 \times 10^2$	$< 5 \times 10^{-5}$	60*
					Added glycine (mg/ml)
145(RP4) × 41	$1{\cdot}7 \times 10^9$	$1{\cdot}1 \times 10^9$	$2{\cdot}2 \times 10^4$	$2{\cdot}0 \times 10^{-5}$	0
	$1{\cdot}1 \times 10^9$	$9{\cdot}1 \times 10^8$	$8{\cdot}2 \times 10^4$	$9{\cdot}0 \times 10^{-5}$	0·19
	$5{\cdot}7 \times 10^8$	$8{\cdot}7 \times 10^8$	$1{\cdot}0 \times 10^5$	$1{\cdot}1 \times 10^{-4}$	0·37
	$1{\cdot}5 \times 10^8$	$3{\cdot}7 \times 10^8$	$2{\cdot}5 \times 10^5$	$6{\cdot}8 \times 10^{-4}$	0·52
	$9{\cdot}3 \times 10^6$	$5{\cdot}4 \times 10^7$	$6{\cdot}6 \times 10^4$	$1{\cdot}2 \times 10^{-3}$	0·74
	$6{\cdot}7 \times 10^6$	$2{\cdot}0 \times 10^7$	$2{\cdot}9 \times 10^4$	$1{\cdot}4 \times 10^{-3}$	0·94

* Plate counts after growth in 50 % or higher levels of NB were more variable than expected from errors in dilution and plating.

was regained. A similar effect was observed by Grinsted *et al.* (1972), who showed that *E. coli* strain W3110(RP1) produced about 50 times more β-lactamase than *Proteus mirabilis* strain Pr-1 carrying the same R factor. Differences in the level of R-TEM mediated β-lactamase activity in *E. coli* and *P. mirabilis* have also been reported by Smith (1969) and Hesslewood & Smith (1974). Though β-lactamase levels were not measured in *R. leguminosarum* these results would suggest that impaired phenotypic expression, rather than genetic alteration of the plasmids, was responsible for the level of resistance observed.

Transfer of R factors was tested in crosses between single donors and recipients and also with mixed *E. coli–R. leguminosarum* recipient cultures. Mixed recipients were used to give a direct comparison between the frequency of inter- and intra-generic transfer of the plasmids under the same mating conditions and also to be certain that R^+ donors were able to transfer the plasmids under these conditions. Using mixed recipients a number of crosses were performed to detect transfer of the C group R factors R40a and R57b, the F-like R factor R1*drd*19 and the I-like R factor R144*drd*3 from *E. coli* to *R. leguminosarum*. Transfer of R40a and R57b was tested as it has been reported that R40a was transmissible from *Pseudomonas aeruginosa* to *E. coli* (Datta & Hedges, 1972*b*); R1*drd*19 and R144*drd*3 were tested as they transfer at high frequency between *E. coli* strains and have been used to mediate chromosomal transfer between members of the Enterobacteriaceae (Cooke & Meynell, 1969; Dixon & Postgate, 1971; Moody & Hayes, 1972). None of these plasmids was transferred to *R. leguminosarum* at a detectable level (10^{-9} or above), though transfer to the *E. coli* recipients in these crosses was always observed.

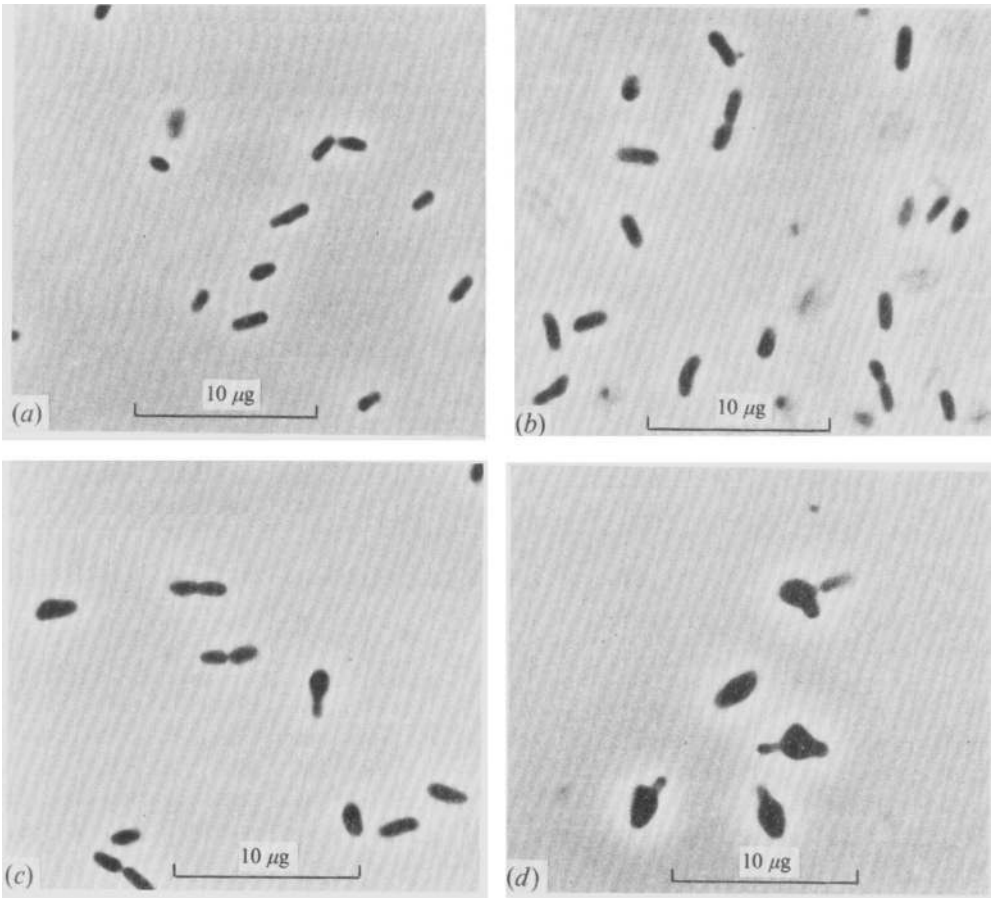

Fig. 1. Effect of (*a*) 0, (*b*) 30, (*c*) 40 and (*d*) 50 % of NB in VS medium on the morphology of *R. leguminosarum* strain 107 after 20 h incubation at 28 °C.

Effects of medium composition on R factor transfer

Transfer of RP4 between *R. leguminosarum* strains in liquid medium (VS) crosses occurred less frequently than between *E. coli* or *P. aeruginosa* strains crossed in nutrient broth (NB). As rhizobia are inhibited by high levels of amino acids (Jordan & Coulter, 1965; Skinner & Roughley, 1969), mating could not be performed in NB; therefore crosses were made in liquid media containing different proportions of VS and NB to determine whether a rich medium was advantageous.

NB had two main effects on R factor transfer (Table 3). Low levels apparently enriched the medium and enabled the bacteria to reach a higher final density after 20 h incubation, and this also had the effect of increasing the yield of R^+ recipients. The frequency of transfer was also stimulated by increased levels of NB up to a point where the yield of donors and recipients was severely reduced. This point, about 40 to 50 % NB, also corresponded to the point where swollen and distorted bacteria could first be observed (Fig. 1).

Sherwood (1972) reported that the inhibition and morphological changes induced in rhizobia by high levels of yeast extract were mainly due to the glycine present. Therefore, crosses were performed in VS containing different amounts of glycine to test whether the effect of NB on R factor transfer in *R. leguminosarum* was due to the provision of essential

Table 4. *Transfer of RP4 in plate crosses*

Plate crosses were performed as described in Methods, selecting for RP4 transfer with kanamycin and counter-selecting donors by their growth requirements.

Donor	No. of isolates tested for recipient ability	No. of isolates that received RP4
41(RP4)	63	51
107(RP4)	52	25
145(RP4)	70	50
147(RP4)	65	30

Table 5. *Lack of effect of DNase on R factor transfer*

Crosses were performed as described in Methods, in 40 % NB+60 % VS with and without DNase at 40 μg/ml.

	Viable count (per ml of culture)					
	+DNase*			−DNase		
Cross	R^+ Donors	Recipients	R^+ recipients	R^+ donors	Recipients	R^+ recipients
145(RP4)×41	$2{\cdot}6\times10^9$	$1{\cdot}6\times10^9$	$1{\cdot}9\times10^4$	$7{\cdot}1\times10^8$	$1{\cdot}3\times10^9$	$1{\cdot}2\times10^4$
41(RP4)×145	$1{\cdot}2\times10^9$	$3{\cdot}1\times10^9$	$3{\cdot}5\times10^3$	$8{\cdot}5\times10^8$	$8{\cdot}2\times10^8$	$1{\cdot}6\times10^3$

* DNase (1 ml) at 400 μg/ml in 0·2 M-$MgSO_4$, was added to 9 ml of mating medium.

metabolites required for conjugation or to the glycine it contained. Transfer was stimulated by the addition of glycine and the effect of glycine was similar to that with NB (Table 3). As with NB, the level which was responsible for a reduced yield of donors and recipients was found to be the one where morphological changes in the bacteria could be observed.

Host range for RP4 transfer

To determine whether R factors could be transferred to a wide range of *R. leguminosarum* strains, four different R^+ donors were crossed with a large number of distinct 'wild-type' isolates (Table 4). Of 52 isolates plate crossed with all four donors, only five were found that would not act as recipients of RP4 from any of the donors. No obvious differences, such as bacteriocin production or sensitivity, could be determined between these strains and those which acted as recipients. The data show a widespread ability of *R. leguminosarum* strains to act as recipients of RP4 in crosses of this type.

Lack of effect of deoxyribonuclease on R factor transfer between R. leguminosarum strains

If transfer of R factors between *R. leguminosarum* strains were due to spontaneous transformation, the presence of deoxyribonuclease (DNase) in the mating medium would prevent, or at least lower, the frequency of transfer. Crosses were performed in the presence and absence of DNase (Koch Light, bovine pancreas) under conditions giving optimal levels of R factor transfer (Table 5). The data show that transfer was not inhibited by DNase.

Stability of R factors

Stanisich & Holloway (1971) reported that R6886 was unstable in *Pseudomonas aeruginosa* strain PAT and that the tetracycline and kanamycin/neomycin determinants of R1822 were

lost on transfer of this R factor between *P. aeruginosa* strains. Grinsted *et al.* (1972) observed that this separation of antibiotic resistance markers could occur spontaneously with RP1 in *P. aeruginosa* and Ingram *et al.* (1972) suggested that this might be a result of integration of the carbenicillin resistance determinant into the host chromosome. Furthermore, Olsen & Shipley (1973) reported that R1822 was unstable in strains of *Neisseria perflava*, *Rhodospirillum rubrum* and *Rhodopseudomonas spheroides*.

Three R^+ *R. leguminosarum* strains were tested for the stability of the plasmids they carried by repeatedly subculturing on antibiotic-free TY agar and then plating for single colonies on antibiotic-free SY agar. These colonies were then replicated to plates of SY medium containing kanamycin, tetracycline or carbenicillin. I examined 1147 colonies of strain 145(RP4), 1649 colonies of strain 145(R6886) and 1620 colonies of strain 145(RK2), but detected no separation of resistance determinants or loss of R factors. No loss of R factor markers has been observed from any other *R. leguminosarum* strain in this laboratory.

R factor mediated recombination

Chromosomal gene transfer mediated by R factors in members of the Enterobacteriaceae has been well documented (Cooke & Meynell, 1969; Dixon & Postgate, 1971; Moody & Hayes, 1972) and has also been demonstrated in *P. aeruginosa* using R factors of the compatibility class P (Stanisich & Holloway, 1971). Crosses between *R. leguminosarum* strains were performed to look for recombinant formation associated with R factor transfer. These crosses were performed as described in Methods using 40% NB+60% VS as the mating medium; selection was for R factor transfer and recombinant formation for individual auxotrophic markers of both parents. In over 30 crosses of this type, involving transfer of RP4, R6886, RK2 and R751 between suitable pairs of the *R. leguminosarum* strains described in Methods, no increased frequency of prototroph formation over the spontaneous mutation rate (usually less than 10^{-8}) was observed for any marker tested, though R factor transfer occurred at a frequency of 10^{-3} to 10^{-5}.

DISCUSSION

These results show that *R. leguminosarum*, like many other Gram-negative bacteria (Datta *et al.* 1971; Datta & Hedges, 1972*a*; Olsen & Shipley, 1973), can act as a recipient or donor of P group R factors in mixed culture with other bacteria. The insensitivity of this transfer to deoxyribonuclease, the wide range of *R. leguminosarum* strains that are able to act as recipients of R factors, and the observation that transfer can occur both to and from *E. coli*, make conjugation the most likely mode of transfer. Therefore, as was originally anticipated, R factor transfer experiments have provided a method for demonstrating conjugation in *R. leguminosarum*.

An unusual feature of crosses involving *R. leguminosarum* strains was the effect of NB or glycine on the frequency of R factor transfer. These crosses indicated that, in the presence of high levels of amino acids or glycine in the mating medium, the bacteria were unable to grow and divide normally and, therefore, when samples from a cross were plated on selective media the number of donors and recipients recovered from the mating medium declined. Furthermore, the frequency of R factor transfer was stimulated by added glycine or NB up to the point where growth was severely affected. A reasonable interpretation of these results is that high levels of glycine (and possibly other amino acids present in NB) in the medium induce spheroplast formation and interfere with cell division. The formation of partial spheroplasts at low levels of NB could have a similar beneficial effect on mating

properties to that observed with some bacteria as a result of mutation from the 'smooth' to the 'rough' phenotype (Jarolmen & Kemp, 1969; Heumann, 1968) as in both cases changes in the outer layers of the bacteria are involved. This interpretation is consistent with the following: high levels of glycine are known to induce spheroplast formation in bacteria (McQuillen, 1960; Strominger & Birge, 1965); rhizobia are sensitive to high levels of glycine and yeast extract in media and these cause spheroplast formation (Jordan & Coulter, 1965; Sherwood, 1972). Examination of mating mixtures containing different levels of NB or glycine in the light microscope showed that the optimum frequency of R factor transfer occurred at a level of NB or glycine that induced obvious morphological changes in the bacteria. Having observed this effect, future crosses were performed in a medium containing 40% NB:60% VS.

The inability to demonstrate transfer of chromosomal markers mediated by R factor transfer was not unexpected, in comparison with R factor mediated recombination in the Enterobacteriaceae (Pearce & Meynell, 1968; Cooke & Meynell, 1969; Dixon & Postgate, 1971). In these well-characterized systems the frequency of recombinant formation for most chromosomal markers is about 10^5 times lower than that for transfer of the R factor. Therefore, de-repressed transfer mutants able to transfer at approximately 100% efficiency are needed to give reasonable numbers of recombinants. Transfer of P group R factors between *R. leguminosarum* strains was at approximately the same level as that of repressed R factors in the Enterobacteriaceae and it can therefore be assumed that chromosomal mobilization might well have been too infrequent to be observed. The recent report by Cole & Elkan (1973) that *Rhizobium japonicum* carries extrachromosomal antibiotic resistance genes, and that these can be transferred by conjugation to *Agrobacterium tumefaciens*, suggests that future studies of conjugation in *R. leguminosarum* would benefit from a search for self-transmissible plasmids that might already be present in Rhizobium strains.

I am grateful to Professor D. A. Hopwood for his advice and assistance during the preparation of this paper, and to those who so readily provided me with bacterial cultures.

REFERENCES

Balassa, G. (1963). Genetic transformation of *Rhizobium:* A review of the work of R. Balassa. *Bacteriological Reviews* **27**, 228–241.

Cartwright, P. M. (1967). The effect of combined nitrogen on the growth and nodulation of excised roots of *Phaseolus vulgaris*. *Annals of Botany* N.S. **31**, 309–321.

Clowes, R. C. & Hayes, W. (1968). *Experiments in Microbial Genetics*. Oxford and Edinburgh: Blackwell Scientific Publications.

Cole, M. A. & Elkan, G. H. (1973). Transmissible resistance to penicillin G, neomycin, and chloramphenicol in *Rhizobium japonicum*. *Antimicrobial Agents and Chemotherapy* **4**, 248–253.

Cooke, M. & Meynell, E. (1969). Chromosomal transfer mediated by de-repressed R factors in F⁻ *Escherichia coli* K12. *Genetical Research* **14**, 79–87.

Cooke, M., Meynell, E. & Lawn, A. M. (1970). Mutant Hfr strains defective in transfer: restoration by F-like and I-like de-repressed R factors. *Genetical Research* **16**, 101–112.

Datta, N. & Hedges, R. W. (1972*a*). Host ranges of R factors. *Journal of General Microbiology* **70**, 453–460.

Datta, N. & Hedges, R. W. (1972*b*). R factors identified in Paris, some conferring gentamycin resistance, constitute a new compatibility group. *Annales de l'Institut Pasteur* **123**, 849–852.

Datta, N., Hedges, R. W., Shaw, E. J., Sykes, R. B. & Richmond, M. H. (1971). Properties of an R factor from *Pseudomonas aeruginosa*. *Journal of Bacteriology* **108**, 1244–1249.

Dixon, R. A. & Postgate, J. R. (1971). Transfer of nitrogen-fixation genes by conjugation in *Klebsiella pneumoniae*. *Nature, London* **234**, 47–48.

Dudman, W. F. (1964). Growth and extracellular polysaccharide production by *Rhizobium meliloti* in defined medium. *Journal of Bacteriology* **88**, 640–645.

GRINSTED, J., SAUNDERS, J. R., INGRAM, L. C., SYKES, R. B. & RICHMOND, M. H. (1972). Properties of an R factor which originated in *Pseudomonas aeruginosa* 1822. *Journal of Bacteriology* **110**, 529–537.

HESSLEWOOD, S. R. & SMITH, J. T. (1974). Inhibition of R-factor-mediated beta-lactamase by *Proteus mirabilis*. *Antonie van Leeuwenhoek* (in the Press).

HEUMANN, W. (1968). Conjugation in star-forming *Rhizobium lupini*. *Molecular and General Genetics* **102**, 132–144.

HEUMANN, W., PÜHLER, A. & WAGNER, E. (1971). The two transfer regions of the *Rhizobium lupini* conjugation. I. Fertility factor elimination and one way transfer. *Molecular and General Genetics* **113**, 308–315.

HEUMANN, W., PÜHLER, A. & WAGNER, E. (1973). The two transfer regions of the *Rhizobium lupini* conjugation. II. Genetic characterization of the transferred chromosomal segments. *Molecular and General Genetics* **126**, 267–274.

HOLLOWAY, B. W. & RICHMOND, M. H. (1973). R factors used for genetic studies in strains of *Pseudomonas aeruginosa* and their origin. *Genetical Research* **21**, 103–105.

HOPWOOD, D. A., HAROLD, R. J., VIVIAN, A. & FERGUSON, H. M. (1969). A new kind of fertility variant in *Streptomyces coelicolor*. *Genetics* **62**, 461–477.

INGRAM, L., SYKES, R. B., GRINSTED, J., SAUNDERS, J. R. & RICHMOND, M. H. (1972). A transmissible resistance element from a strain of *Pseudomonas aeruginosa* containing no detectable extrachromosomal DNA. *Journal of General Microbiology* **72**, 269–279.

JAROLMEN, H. & KEMP, G. (1969). Association of increased recipient ability for R factors and reduced virulence among variants of *Salmonella choleraesuis* var. *kunzendorf*. *Journal of Bacteriology* **97**, 962–963.

JOBANPUTRA, R. S. & DATTA, N. (1974). Trimethoprim resistance factors in enterobacteria from clinical specimens. *Journal of Medical Microbiology* (in the Press).

JORDAN, D. C. & COULTER, W. M. (1965). On the cytology and synthetic capacities of natural and artificially produced bacteriods of *Rhizobium leguminosarum*. *Canadian Journal of Microbiology* **11**, 709–720.

KAUDEWITZ, F. (1959). Production of bacterial mutants with nitrous acid. *Nature, London* **183**, 1829–1830.

KOWALSKI, M. (1971). Transduction in *Rhizobium meliloti*. *Plant and Soil, Special Volume*, pp. 63–66.

LAWN, A. M. & MEYNELL, E. (1970). Serotypes of sex pili. *Journal of Hygiene* **68**, 683–694.

MCQUILLEN, K. (1960). Bacterial protoplasts. In *The Bacteria*, vol. 1, pp. 249–359. Edited by I. C. Gunsalus and R. Y. Stanier. New York: Academic Press.

MOODY, E. E. M. & HAYES, W. (1972). Chromosome transfer by autonomous transmissible plasmids: the role of the bacterial recombination (*rec*) system. *Journal of Bacteriology* **111**, 80–85.

OLSEN, R. H. & SHIPLEY, P. (1973). Host range and properties of the *Pseudomonas aeruginosa* R factor R1822. *Journal of Bacteriology* **113**, 772–780.

PEARCE, L. E. & MEYNELL, E. (1968). Specific chromosomal affinity of a resistance factor. *Journal of General Microbiology* **50**, 159–172.

PÜHLER, A., BURKARDT, H. J. & HEUMANN, W. (1972). Genetic experiments with the *Pseudomonas aeruginosa* R factor RP4 in *Rhizobium lupini*. *Journal of General Microbiology* **73**, xxvi.

RAINA, J. L. & MODI, V. V. (1972). Deoxyribonucleate binding and transformation in *Rhizobium japonicum*. *Journal of Bacteriology* **111**, 356–360.

ROE, E., JONES, R. J. & LOWBURY, E. J. L. (1971). Transfer of antibiotic resistance between *Pseudomonas aeruginosa*, *Escherichia coli* and other Gram-negative bacilli in burns. *Lancet* i, 149–152.

SCHERRER, A. (1970). *Étude des propriétés symbiotiques de certains mutants auxotrophs de Rhizobium meliloti*. Faculté des Sciences d'Orsay, D. E. A. d'Amélioration des Plantes, octobre 1970.

SERMONTI, G. & CASCIANO, S. (1963). Sexual polarity in *Streptomyces coelicolor*. *Journal of General Microbiology* **33**, 292–301.

SHERWOOD, M. T. (1970). Improved synthetic medium for the growth of *Rhizobium*. *Journal of Applied Bacteriology* **33**, 708–713.

SHERWOOD, M. T. (1972). Inhibition of *Rhizobium trifolii* by yeast extracts or glycine is prevented by calcium. *Journal of General Microbiology* **71**, 351–358.

SIK, T. & OROSZ, L. (1971). Chemistry and genetics of *Rhizobium meliloti* phage 16–3. *Plant and Soil, Special Volume*, pp. 57–62.

SKINNER, F. A. & ROUGHLEY, R. J. (1969). *Rothamsted Report* Part 1, pp. 101–102.

SMITH, J. T. (1969). R factor gene expression in Gram-negative bacteria. *Journal of General Microbiology* **55**, 109–120.

STANISICH, V. A. & HOLLOWAY, B. W. (1971). Chromosome transfer in *Pseudomonas aeruginosa* mediated by R factors. *Genetical Research* **17**, 169–172.

STROMINGER, J. L. & BIRGE, C. H. (1965). Nucleotide accumulation induced in *Staphylococcus aureus* by glycine. *Journal of Bacteriology* **89**, 1124–1127.

SYKES, R. B. & RICHMOND, M. H. (1970). Intergeneric transfer of a beta-lactamase gene between *Ps. aeruginosa* and *E. coli*. *Nature, London* **226**, 952–954.

VINCENT, J. M. (1970). In *A Manual for the Practical Study of the Root-nodule Bacteria*. I.B.P. Handbook no. 15. Oxford and Edinburgh: Blackwell Scientific Publications.

VOGEL, H. J. & BONNER, D. M. (1956). Acetylornithinase of *Escherichia coli*: partial purification and some properties. *Journal of Biological Chemistry* **218**, 97–106.