Linkage Mapping in *Rhizobium leguminosarum* by means of R Plasmid-mediated Recombination

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Wild-type R plasmids of the P1 incompatibility group mediated the transfer of chromosomal genes in *Rhizobium leguminosarum*, but only at very low frequencies. Two P1 R plasmids, which had originally been selected in *Pseudomonas aeruginosa* for enhanced donor properties, promoted much higher levels of gene transfer in *R. leguminosarum*. One of these, R68.45, was used for linkage mapping. All markers tested mapped on a single circular linkage group. Segments of donor chromosome up to at least one-seventh of its total length were transferred and integrated into the recipient chromosome.

INTRODUCTION

Plasmids which confer drug resistance on their bacterial hosts (R plasmids) have been used to mediate conjugation and chromosome transfer in many species (Cooke & Meynell, 1969; Dixon, Cannon & Postgate, 1975; Towner & Vivian, 1976; Haas & Holloway, 1976; Beringer & Hopwood, 1976). They have the advantage that their transfer can be selected by means of the antibiotic resistance that they confer on their new host. Of particular interest are R plasmids of the P1 incompatibility group because they have very wide host ranges among Gram-negative bacteria (Datta *et al.*, 1971; Olsen & Shipley, 1973). They have been used to promote chromosome transfer in bacterial species which were not known to carry indigenous sex factors, such as *Acinetobacter calcoaceticus* (Towner & Vivian, 1976), *Pseudomonas glycinea* (Lacey & Leary, 1976) and *Rhizobium* spp. (Beringer & Hopwood, 1976; Meade & Signer, 1977; Johnston & Beringer, 1977; Kondorosi *et al.*, 1977).

We report here some of the characteristics of R plasmid-mediated recombination in *Rhizobium leguminosarum* and show that this organism has a single circular linkage group.

METHODS

Bacterial strains and plasmids are shown in Table 1.

Media. Complete (TY) and minimal (Y) media for culturing *R. leguminosarum* and mixtures of *R. leguminosarum* with *Escherichia coli* or *Pseudomonas aeruginosa* were described by Beringer (1974). For culturing *E. coli* and *P. aeruginosa* strains alone, Oxoid Nutrient Broth (NB) was the complete medium; it was solidified, when required, with Lab M agar (15 g l⁻¹; London Analytical and Bacteriological Media, London). The minimal medium was that of Vogel & Bonner (1956) containing 1.5 % (w/v) Difco agar.

Culture conditions. *Rhizobium leguminosarum* strains and mixtures with *E. coli* or *P. aeruginosa* were cultured at 28 °C; *E. coli* and *P. aeruginosa* strains were cultured alone at 37 °C.

Individual strains of *R. leguminosarum* were prepared for crosses by growing on TY slants in Universal bottles for 2 days. Bacteria were washed off the slants in 20 % (v/v) glycerol and were stored at –20 °C until required. *Escherichia coli* and *P. aeruginosa* strains were cultured overnight in NB, diluted in 9 volumes of fresh warm NB, and cultured for 4 h before crossing with *R. leguminosarum*.

Table 1. *Bacterial strains and plasmids*

Strain no.	Relevant characteristics	Reference or source	
<i>Pseudomonas aeruginosa</i>			
1128	PAO25 (R68.45)	B. W. Holloway	
1124	PAC175 (R68.44)	P. Clarke	
<i>Escherichia coli</i>			
402	j53 <i>nal</i>	E. Meynell	
435	j53 (RP4)	R. W. Hedges	
550	j62 (R702)	R. W. Hedges	
551	402 (R702)	Cross 550 × 402	
1138	402 (R68.44)	Cross 1124 × 402	
1163	j53.2 (R1033)	R. W. Hedges	
<i>Rhizobium leguminosarum</i>			
892	<i>phe-1 ser-1</i>	This paper*	
893	<i>phe-1 ser-1 str-36</i>	This paper*	
1063	<i>ura-14 trp-16 rif-76</i>	This paper*	
1438	<i>ura-14 trp-14 rif-75</i>	This paper*	
952	893 (RP4)	Cross 435 × 893	
1531	893 (R68.45)	Crosses†	
1533	893 (R68.44)	Cross 1138 × 893	
1536	893 (R702)	Cross 551 × 893	
1537	893 (R1033)	Cross 1163 × 893	
Plasmids	Relevant‡ characteristics	Compati- bility	Reference or source
RP4	Ap Km Tc	P1	Datta <i>et al.</i> (1971)
R68.44	Ap Km Tc	P1	Haas & Holloway (1976)
R68.45	Ap Km Tc	P1	Haas & Holloway (1976)
R702	Km Sm Su Tc	P1	Hedges & Jacob (1974)
R1033	Ap Cm Ge Km Sm Su Tc	P1	Smith <i>et al.</i> (1975)

* These strains are all derived from the wild-type strain 300 (Johnston & Beringer, 1975) by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis for the auxotrophic markers and by spontaneous mutation for the antibiotic-resistance markers (Beringer, 1974).

† R68.45 was originally transferred from strain 1128 to *R. leguminosarum* 892 and then successively to three other *R. leguminosarum* strains before being transferred to 893.

‡ Abbreviations for plasmid-mediated antibiotic resistances: Ap, ampicillin; Cm, chloramphenicol; Ge, gentamicin; Km, kanamycin; Sm, streptomycin; Su, sulphonamides; Tc, tetracycline

Crosses were performed as described by Jacob *et al.* (1976). This involved mixing 1 ml cultures of donor and recipient (each containing about 10^9 bacteria ml⁻¹) in about 10 ml distilled water and then sucking the bacteria on to the surface of a Millipore membrane (0.45 µm pore size). For *R. leguminosarum* × *R. leguminosarum* crosses the membranes were placed on the surface of TY plates and incubated for about 20 h at 28 °C. The bacteria were then suspended in 5 ml distilled water, diluted in distilled water, and plated on selective media.

RESULTS

Chromosome mobilization by different P-group plasmids

Derivatives of *R. leguminosarum* strain 893 (*phe-1 ser-1 str-36*) carrying the R plasmids RP4, R1033, R702, R68.44 and R68.45 were used as donors in crosses with *R. leguminosarum* strains 1063 (*ura-14 trp-16 rif-76*) and 1438 (*ura-14 trp-14 rif-75*). Selection was made for Ura⁺, Trp⁺ and Str^r and the frequency of R plasmid transfer was calculated on the basis of the frequency of transfer of kanamycin resistance. The frequency of transfer of R68.44 was about 10⁻¹ per recipient while for the other plasmids it was about 10⁻². The transfer of selected markers by donors carrying RP4, R1033 and R702 was hardly detectable (frequency about 10⁻⁹). The frequency of transfer of selected markers by the R68.44 donor was about 10⁻⁵ while that for the R68.45 donor was about 10⁻⁶. Although this suggested that R68.44 was potentially a better sex plasmid, R68.45 was used in further crosses

because it was derived from R68.44 by Haas & Holloway (1976) on the basis that it was a more stable plasmid.

Chromosome mapping

All the mapping data presented here are from R68.45-mediated crosses. In these crosses, chromosomal markers were usually transferred between *R. leguminosarum* strains at a frequency of about 10^{-6} per donor, and all markers mapped on a single circular linkage group (Fig. 1*a*). Figure 1(*b*) is a linear representation of the linkage map showing the co-inheritance percentages between various pairs of chromosomal markers. The co-inheritance percentage was the number of times per 100 colonies that recombinants initially selected for the inheritance of one chromosomal donor marker were shown to have received a second, non-selected, donor marker during the same cross. All co-inheritance frequencies over 3 % are included to show the variation obtained in different crosses. We routinely scored 150 colonies for any particular selection, so unlinked markers were those with co-inheritance frequencies below 0.7 %. This threshold was sometimes reduced to less than 0.25 % when 400 colonies were analysed (e.g. *trp-16* and *ura-14* in the cross shown in Table 2 showed less than 0.25 % co-inheritance with *str-36*).

In all crosses where both the mutant and the wild-type alleles of the same gene were used as donor markers, each was expressed amongst the recombinants. This is strong evidence that true haploid recombinants were formed.

The relative frequencies of inheritance of different selected markers in a cross almost always varied less than twofold, though in one cross a difference of eightfold was observed. A twofold variation is no greater than would be expected from the different efficiencies of plating (e.o.p.) of *R. leguminosarum* strains on different media (e.g. the e.o.p. on minimal medium plus serine was often 50 % of that on minimal medium alone). This indicates that chromosome mobilization was not restricted to a small number of sites and is in agreement with the results of Meade & Signer (1977) using RP4 in *R. meliloti*.

Size of transferred fragments

The mapping of chromosomal genes in *R. leguminosarum* was done on the basis of co-inheritance frequencies and the distance between markers on the linkage map was drawn on the assumption of a direct proportionality between co-inheritance frequencies and physical distance. The linkage map in Fig. 1(*a*) shows that the circularity of the chromosome can be demonstrated using only seven markers, each of which is co-inherited with adjacent markers at a frequency of 3 to 11 %. Even if such co-inheritance frequencies are not directly proportional to physical distance, some DNA fragments must represent at least one-seventh of the total length of the chromosome. Therefore R68.45 is able to mobilize large fragments of DNA between *R. leguminosarum* strains.

Two extreme possibilities exist concerning the integration of large fragments of DNA into a bacterial chromosome. Both need end 'crossovers' for the fragments to become integrated. The first is that there will be multiple crossovers and genes mapping in the middle of the fragment will tend to segregate independently from those towards the ends. The other is that the whole fragment will become integrated and in this case all donor markers on the fragment should be integrated together. Therefore the amount of crossing-over occurring during integration should be reflected in the segregation of the middle and outer markers on such a fragment.

The types of recombinant formed in a cross where the segregation of a centre marker could be studied when two, not too closely linked, flanking donor markers were selected are shown in Table 2. The data show that when the flanking markers (*ura-14* and *trp-16*) were inherited from the donor the centre donor marker was nearly always inherited with them. For example 88/91 of the *ura⁺trp⁺* recombinants (i.e. the *phe rif* and *wt rif* recombinants from both selections) were also *phe*, which would suggest that crossing-over was relatively infrequent.

Fig. 1(a). Circular linkage map of *R. leguminosarum* strain 300. Numbers on the arrows of the inner circle indicate the co-inheritance frequencies of markers chosen to show circularity using only seven markers with co-inheritance frequencies of 3 to 11 %.

Fig. 1(b). Preliminary linkage map for *R. leguminosarum* strain 300. Numbers above markers represent allele numbers; none are given for *rif* and *str* because many alleles for each marker were studied and all mapped in this region. *rif* and *str* are shown together since linkage between them was usually greater than 90 % and no order in relation to *ade-71* and *cys-8* was determined. Numbers on arrows below the line representing the linkage map are co-inheritance percentages for the alleles shown. Each number was obtained from a different cross. The ordering of markers on the map was determined on the basis of the observed linkage to other markers, as shown on the map, and also on the absence of linkage to other markers.

Table 2. Partial analysis of an R68.45-mediated cross

Cross	1531 × 1063 [<i>phe-1 ser-1 str-36</i> (R68.45) × <i>ura-14 trp-16 rif-76</i>]				
	<i>str-36</i>	<i>ser-1</i>	+	<i>phe-1</i>	+
Relevant map order	<i>rif-76</i>	+	<i>trp-16</i>	+	<i>ura-14</i>
Selected donor marker	Genotype of recombinants in respect of non-selected markers*	No. of recombinants†			
		R ⁺	R ⁻		
<i>ura</i> ⁺	<i>rif phe</i> ⁺ <i>trp</i>	227	54‡		
	<i>rif phe</i> <i>trp</i>	56	5		
	<i>rif phe trp</i> ⁺	46	3		
	<i>rif phe</i> ⁺ <i>trp</i> ⁺	2	0		
<i>trp</i> ⁺	<i>rif phe</i> ⁺ <i>ura</i>	190	49‡		
	<i>rif phe ura</i>	101	20		
	<i>rif phe ura</i> ⁺	36	3		
	<i>rif phe</i> ⁺ <i>ura</i> ⁺	1	0		

* *ser-1*, *str* and *rif* map sufficiently far away from *trp-16* and *ura-14* that no recombinants showing segregation of these markers were obtained.

† 400 colonies were picked from each initial selective medium (phenylalanine plus uracil and phenylalanine plus tryptophan). These colonies were patched on the same medium and replica-plated to diagnostic media to determine their phenotypes and, hence, the segregation of unselected markers. R⁺ and R⁻ indicate whether the recombinants were kanamycin and tetracycline resistant and, therefore, carried R68.45.

‡ Spontaneous revertants or R⁻ recombinants.

Properties of *R. leguminosarum* recombinants

The majority of recombinants arising from R plasmid-mediated crosses were R⁺ (Table 2). This character was used to distinguish recombinants for only a single marker from spontaneous revertants of the recipient, since the latter had only a 1 % chance of being R⁺. When analysing cross data, R⁻ bacteria, which might have been either recombinants or spontaneous mutants, were not included amongst the recombinants. Neither were colonies that differed from the donor on the basis of the change of one marker. This was because they could not be distinguished from revertants and because transfer was strongly polarized from the donors to recipients which made it further unlikely that they would be recombinants.

Recombinants that carried R68.45 were routinely used as donors in further crosses and there was no evidence to suggest that they were generally better chromosome donors than those strains that had received the plasmid directly from *E. coli*. Likewise R⁻ recombinants did not appear to act as better recipients than strains that had not previously been involved in crosses.

DISCUSSION

In this study large fragments of chromosome were shown to be transferred and integrated in *R. leguminosarum* × *R. leguminosarum* crosses with little crossing-over occurring between quite widely separated markers. Johnston & Beringer (1977) reported that a *spc* allele mapping between *phe-1* and *trp-18* (which are about 30 % co-inherited) was always inherited when the flanking markers were transferred together from *R. trifolii* to *R. leguminosarum* and a similar observation was made by Meade & Signer (1977) in *R. meliloti*; 98 % of recombinants receiving two selected donor markers (showing 20 % co-inheritance) also received a marker located between them. These observations may indicate that these species have poor recombination ability, at least when P1-group R plasmids are used to promote chromosome transfer. It could also imply that there is some type of 'end effect' favouring the inheritance of entire transferred fragments due to preferential recombination

towards the end of the fragments. The integration of large segments of linked markers differs from the situation in *E. coli* Hfr-mediated recombination where markers more than 3 min apart (about 1/30 of the chromosome) tend to segregate independently (Hayes, 1968). In *E. coli*, there is physical evidence that the length of each inserted piece of DNA is small [about 250000 to a few million in molecular weight (Siddiqi & Fox, 1973)]; no information on this point is available for *R. leguminosarum*.

The majority of recombinants in *R. leguminosarum* crosses were R⁺, suggesting that gene mobilization does not depend on the formation of Hfr-like donors. However, a sufficiently large number were R⁻ to suggest that Hfr-like donors may occur. It is possible that recombinants preferentially include the progeny of those bacteria that have been involved in a stable mating. If the relatively low frequency of R68.45 transfer to non-recombinants is due to the instability of most mating pairs, that part of the population known to have been involved in stable matings could have received the R plasmid at a greater frequency than the population as a whole. If this were the case, a high proportion of recombinants formed in Hfr-type matings could have become R⁺. If the probability of establishment of a transferred R68.45 plasmid in a new host were less than unity, all recombinants could have arisen from R⁺ donors, the observed R⁻ recombinants simply representing those in which the R plasmid failed to be established.

Chromosome mobilization by F and a number of R plasmids in *E. coli* can arise by integration of the plasmids into the donor chromosome to form Hfr-type donors (Moody & Runge, 1972) or by some other form of interaction not requiring stable integration (Curtiss & Renshaw, 1969; Evenchik, Stacey & Hayes, 1969; Moody & Hayes, 1972). In Hfr × F⁻ crosses, recombinants are normally F⁻ except when the whole chromosome is transferred. In F⁺-mediated crosses recombinants become F⁺; even those derived via rare Hfr donors usually become F⁺ by subsequent infection because of the very high frequency with which F is transferred between *E. coli* strains.

The frequency of R plasmid transfer between *R. leguminosarum* strains was about 10⁻². Therefore, if the R plasmid-mediated recombination were due to the formation of true Hfr-like donors (i.e. those where a significant part of the plasmid remains in the donor unless the whole chromosome is transferred) these recombinants should have had only a low (1 %) probability of being R⁺. The data in Table 2 and for all R plasmid-mediated crosses in *R. leguminosarum* show that the majority of recombinants were R⁺, which suggests that most gene transfer is not of the Hfr type. However, up to 20 % of recombinants that could be unambiguously distinguished from spontaneous revertants (i.e. those where more than one marker had been inherited from each parent) were R⁻ (e.g. the *phe ura rif* and *phe trp rif* recombinants in Table 2) which perhaps indicates that at least some chromosome mobilization may be of the Hfr type.

The observation that R68.44 was more efficient than R68.45 at mobilizing chromosomal genes between *R. leguminosarum* species appears to contradict the previous report of Beringer & Hopwood (1976) and indicate a problem associated with the handling of derivatives of R68 with enhanced chromosome donor properties. Haas & Holloway (1976) and Kondorosi *et al.* (1977) reported that strains carrying these R plasmids could lose their enhanced donor properties. Similarly, seven of the 23 donors that we tested showed normal levels of R plasmid transfer but had lost the enhanced chromosome donor properties of R68.45. This may be the explanation for the poor donor properties of R68.44 reported by Beringer & Hopwood (1976), since they used a different donor strain from that used in the crosses reported here. R⁺ strains have not been found to lose any plasmid-mediated antibiotic resistances nor the plasmids themselves (as judged by the ability to transfer such resistances).

The particular interest of genetic studies in *Rhizobium* spp. is to facilitate our understanding of their nitrogen-fixing symbiosis with leguminous plants. Genetic mapping is important for determining the number and relationship of microbial symbiotic genes.

Mutants in such genes need to be assayed by plant tests, severely limiting the number of recombinants that can be tested. An advantage of the relatively short linkage maps of *R. leguminosarum* and *R. meliloti* (Meade & Signer, 1977; Kondorosi *et al.*, 1977) is that only a few crosses need to be done to arrive at an approximate map location for any new mutations.

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