JOURNAL OF BACTERIOLOGY, Jan. 1982, p. 59–64
0021-9193/82/010059-06$02.00/0

Vol. 149, No. 1

Use of Plasmid R68.45 for Constructing a Circular Linkage Map of the *Rhizobium trifolii* Chromosome

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Received 10 March 1981/Accepted 29 June 1981

Plasmid R68.45 was used to promote conjugal transfer of chromosomal markers in *Rhizobium trifolii* RS55. Analysis of two-factor and three-factor crosses among *R. trifolii* strains enabled construction of a circular linkage map of the *R. trifolii* chromosome, containing 17 nutritional and resistance markers.

The economic advantages that may accrue from the improvement of nitrogen-fixing systems have become increasingly evident during the past decade. One of the most interesting nitrogen-fixing systems is the *Rhizobium*-legume symbiosis because of its high agricultural importance. The past several years have witnessed important developments in *Rhizobium* genetics (3, 7), and a number of suitable procedures for the genetic manipulation of rhizobia have recently been developed (4–6, 8, 9, 17, 18, 20, 23, 24).

For many years, one of the major limitations for genetic analysis of rhizobia was the lack of known indigenous chromosome-mobilizing plasmids that would allow gene transfer studies. This difficulty has been overcome by transferring broad-host-range plasmids with chromosome-mobilizing ability (Cma) to *Rhizobium* spp.

To date, the *Pseudomonas aeruginosa* drug-resistance plasmids R68.45 (13) and RP4 (12) have been the plasmids of choice for obtaining gene transfer in *Rhizobium*. R68.45 is very efficient for mobilizing chromosomal markers in *R. meliloti* (10, 21) and *R. leguminosarum* (5) as well as in other gram-negative bacteria (14, 26). In both *R. meliloti* and *R. leguminosarum*, R68.45 promotes nonpolarized chromosomal transfer from a number of origins (4, 5, 10, 21). In those species of *Rhizobium* so far examined, R68.45 can transfer fragments of chromosome long enough to enable accurate mapping of a variety of markers and to establish chromosome circularity (5, 10, 21).

RP4 is a less efficient sex factor (15). However, it has been successfully used for mediating transfer of chromosomal markers in *R. meliloti* (22).

To date, the only report on linkage mapping studies in *R. trifolii* was an attempt at using comutation techniques (29). As an alternative approach, we describe here the use of plasmid R68.45 for promoting chromosomal transfer in *R. trifolii*. Linkage mapping experiments reported here have led to the construction of a circular linkage map of *R. trifolii* containing 17 nutritional and resistance markers.

MATERIALS AND METHODS

Bacterial strains. The strains used are listed in Table 1.

Plasmid. R68.45, belonging to the P1 incompatibility group and mediating resistance to ampicillin, kanamycin, and tetracycline, was used (13).

Media and growth conditions. *Escherichia coli* was cultured on nutrient broth (Oxoid); when required in solidified form, agar (Oxoid) was added at 10 g/liter.

For *R. trifolii* strains complete medium was YT (2) and minimal medium was that of Vogel and Bonner (27) supplemented with 19 g of glucose per liter and 12 μg of biotin per ml. When required, amino acid and base supplements were used at a final concentration of 1 mM. *E. coli* was always grown at 35°C. *R. trifolii* was always grown at 28°C.

Antibiotics. Except for rifampin, which was dissolved in methanol and added unfiltered, antibiotics were added to agar media as filter-sterilized aqueous solutions. Concentrations used were as follows: streptomycin sulfate (Sigma), 1 mg/ml; kanamycin sulfate (Sigma), 25 μg/ml; oxytetracycline (Sigma), 10 μg/ml; rifampin (Lepetit), 20 μg/ml; ampicillin (Sigma), 10 μg/ml, and canavanine sulfate (Sigma), 80 μg/ml.

Matings. Matings were performed on Sartorius filters (0.45-μm pore size) as described by Jacob et al. (16). One milliliter of log-phase donor and 1 ml of stationary-phase recipient (both containing at least 10^8 cells per ml) were mated on the membrane. Membranes were placed on the surface of nutrient agar plates and incubated for about 20 h at 28°C. Then mating mixtures were resuspended in 5 ml of Tris-salts buffer, pH 7.2, diluted, and plated on selective media.

Linkage mapping of the *R. trifolii* chromosome. Recombinant colonies for a selected marker were picked onto selective media to determine coheritance of unselected markers. Each pair of markers was analyzed by scoring 150 to 300 colonies. It was assumed that linkage values were inversely related to

TABLE 1. Bacterial strains[a]

Strain	Relevant characteristics	Reference or source
E. coli 1230	Pro$^-$ Met$^-$ (R68.45)	J. E. Beringer
R. trifolii		
RS55	Wild-type	Isolated from clover root nodules
RS176	*str-1*	This paper
RS225	*his-6 str-1*	This paper
RS235	*his-6 met-36 str-1*	This paper
RS238	*his-6 phe-18 str-1*	This paper
RS241	*rif-6 ade-17*	This paper
RS246	*rif-6 ade-29*	This paper
RS271	*str-1 leu-23*	This paper
RS288	*his-6 tyr-28 str-1*	This paper
RS290	*his-6 thr-14 str-1*	This paper
RS292	*his-6 pdx-3 str-1*	This paper
RS293	*rif-6 ade-29 tyr-13*	This paper
RS294	*rif-6 ade-29 thr-27*	This paper
RS295	*rif-6 ade-29 arg-19*	This paper
RS296	*his-6 met-31 str-1 can-3*	This paper
RS230 (R68.45)	*his-6 str-1*	Cross 1230 × RS225
RS278 (R68.45)	*rif-6 ade-17*	Cross 1230 × RS241

[a] Allele numbers are arbitrary. Gene symbols are those of Bachmann and Low (1). All the *R. trifolii* strains are derived from the wild-type RS55, isolated in our laboratory from *Trifolium repens* L. nodules. Auxotrophic mutations were induced by either *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine or ethyl methane sulfonate mutagenesis. Nitrosoguanidine treatments were carried out as follows: a log culture containing about 10^8 cells per ml was cooled, washed twice with Tris-maleate buffer, pH 7.5, and resuspended in 500 mg of nitrosoguanidine (Sigma) per liter for 30 min. Then the cells were harvested and carefully washed with buffer before plating on YT agar. For ethyl methane sulfonate mutagenesis, a culture containing about 4×10^8 cells per ml was washed twice with phosphate buffer, pH 8.0, and suspended in 0.1 M ethyl methane sulfonate (Sigma). After incubation at 30°C for 45 min, 6% (wt/vol) $Na_2S_2O_3$ was added. Cells were harvested, washed, and plated on YT. Drug-resistance mutations were spontaneous.

physical distances between genes. Linkage values were transformed into additive map distances by using the equation derived by Kemper (19):

$$C = (1 - t) + t(\ln t)$$

where C is the linkage frequency value and t is the map distance.

RESULTS

Transfer of plasmid R68.45 from *E. coli* to *R. trifolii*. Plasmid R68.45 was easily transferred from *E. coli* 1230 to *R. trifolii* RS176 in membrane matings. Transconjugants were selected on minimal medium with antibiotics. When the recipient strain was one of the auxotrophic derivatives of RS176 described above, selective medium was supplemented with the requirements of the recipient strain.

In these crosses the frequency of plasmid transfer was about 10^{-3} per donor bacterium. This frequency is slightly lower than those re-

TABLE 2. R68.45 transfer and chromosome mobilization in *R. trifolii* RS176

Donor	Recipient	Frequency of R transfer[a]	Selected phenotype	Frequency of recombinants[a]
RS230	RS241	2.8×10^{-1}	Ade$^+$	2.7×10^{-4}
			Str^r	8.9×10^{-4}
RS230	RS294	4.3×10^{-1}	Thr$^+$	1.1×10^{-4}
			Ade$^+$	1.1×10^{-5}
RS278	RS235	4.2×10^{-1}	Met$^+$	3.0×10^{-5}
			His$^+$	9.2×10^{-4}
RS278	RS288	8.3×10^{-1}	His$^+$	1.1×10^{-4}
			Tyr$^+$	1.6×10^{-4}
			Rif^r	6.0×10^{-5}
RS278	RS271	5.8×10^{-1}	Leu$^+$	1.1×10^{-4}
			Rif^r	7.0×10^{-4}
RS278	RS292	3.3×10^{-1}	Pdx$^+$	1.7×10^{-4}

[a] Per donor bacterium.

TABLE 3. Detection of R68.45 markers in transconjugants that had received chromosomal markers

Cross	Selected phenotype	No. of clones tested[a]	R^+	R^-	% R
RS230 × RS293	Ade^+	204	198	6	3
RS278 × RS288	Tyr^+	163	160	3	2
RS278 × RS238	His^+	113	112	1	1
RS230 × RS271	Str^r	199	197	2	1

[a] Replica plated on antibiotic medium.

ported for *E. coli* × *R. leguminosarum* and *E. coli* × *R. meliloti* crosses (5, 10).

From these crosses R^+ transconjugants of *R. trifolii* were isolated to be used as further donors in intraspecific crosses. All isolates were purified two or three times before being stored.

Intraspecific matings: transfer of plasmid R68.45 and mobilization of chromosomal markers in *R. trifolii*. As shown in Table 2, the frequency of plasmid transfer in intraspecific matings was higher than in intergeneric crosses. Transfer frequencies described here are similar to those reported for other *Rhizobium* species, such as *R. meliloti* (9, 21), *R. leguminosarum* (4, 5), and interspecific crosses of *R. leguminosarum* × *R. meliloti* (17).

Mobilization of single chromosomal markers occurred at frequencies ranging from about 5×10^{-5} to 5×10^{-3} per donor cell. Absence of great differences among the frequencies of mobilization of different markers indicated that R68.45 was able to promote chromosome transfer at a variety of origin sites as previously reported for other rhizobia (4, 5, 10, 21).

When the presence of plasmid R68.45 was investigated among the recombinants, most of them proved to be R^+ at least for resistance markers (see Table 3). This suggested that chromosomal transfer mediated by plasmid R68.45 was F'-like rather than Hfr-like. The same phenomenon has been described in *R. leguminosarum* and *R. meliloti* (4, 10, 21).

Linkage analysis. The rationale for linkage studies based upon R68.45-mediated gene transfer can be summarized as follows: since any marker has a relatively defined frequency of transfer, the chance of a pair of markers being cotransferred in the same cross will depend on the distance between them.

Table 4 shows the results obtained in a series of two-factor crosses. In each mating initial selection was carried out for a single marker.

TABLE 4. Linkage analysis of several marker pairs of *R. trifolii* RS176

Donor	Recipient	Selected phenotype	Markers pairs	Linkage (*C*)	Distance (*t*)
RS230	RS246	Rif^r	*rif-6 ade-29*	0.41	0.24
			rif-6 str-1	0.21	0.42
		Ade^+	*ade-29 str-1*	0.04	0.73
RS230	RS293		*ade-29 tyr-13*	0.39	0.26
		Rif^r	*rif-6 tyr-13*	0.79	0.05
		Tyr^+	*tyr-13 str-1*	0.17	0.47
RS230	RS294	Thr^+	*thr-18 ade-29*	0.75	0.06
			thr-18 rif-6	0.69	0.09
RS230	RS295	Ade^+	*ade-29 arg-19*	0.65	0.10
		Rif^r	*rif-6 arg-19*	0.25	0.38
RS278	RS235	His^+	*his-6 met-36*	0.40	0.25
		Met^+	*met-36 rif-6*	0.03	0.76
RS278	RS238	Phe^+	*phe-18 his-6*	0.04	0.73
			phe-18 rif-6	0.16	0.49
RS278	RS271	Leu^+	*leu-23 rif-6*	0.04	0.73
		Ade^+	*ade-29 leu-23*	0.47	0.20
RS278	RS290	Thr^+	*thr-27 his-6*	0.42	0.23
		Str^r	*str-1 thr-27*	0.09	0.60
		Rif^r	*rif-6 thr-27*	0.70	0.08
RS278	RS292	Pdx^+	*pdx-3 str-1*	0.27	0.36
			pdx-3 his-6	0.03	0.76
RS278	RS296	Can^r	*can-3 his-6*	0.16	0.49
			can-3 met-31	0.54	0.16
		His^+	*his-6 met-31*	0.35	0.29
		Met^+	*met-31 rif-6*	0.12	0.55

TABLE 5. Three-factor crosses between *R. trifolii* strains

Donor	Recipient	Selected phenotype	Markers pairs	Linkage (*C*)	Distance (*t*)
RS278	RS288	His^+	*his-6 tyr-28*	0.43	0.23
			his-6 ade-17	0.06	0.67
		Tyr^+	*tyr-28 his-6*	0.46	0.21
			tyr-28 ade-17	0.28	0.35
RS278	RS238	His^+	*his-6 phe-18*	0.05	0.70
			his-6 ade-17	0.07	0.65
		Phe^+	*phe-18 his-6*	0.06	0.67
RS278	RS271	Str^r	*str-1 ade-17*	0.19	0.45
		Leu^+	*leu-23 ade-17*	0.03	0.76
RS230	RS293	Tyr^+	*tyr-13 ade-29*	0.28	0.35
			tyr-13 his-6	0.23	0.40
		Ade^+	*ade-29 tyr-13*	0.26	0.37
			ade-29 his-6	0.03	0.76

Cotransfer of unselected markers was detected by replica plating on appropriate selective media. Linkage frequencies lower than 0.03 were discarded. Distances were calculated by using the Kemper equation as described above.

Results obtained in three-factor crosses are summarized in Table 5.

A diagram that summarizes the linkage relationships among 17 chromosomal markers is presented in Fig. 1. Numbers above the arrows indicate linkage frequencies found in independent experiments. This preliminary linkage map can be circularized and reproduced to scale as shown in Fig. 2.

DISCUSSION

Mobilization of the *R. trifolii* chromosome can be achieved by using several plasmids, such as RP1, RP4, R1drd19, and R68.45 (M. Megías, Ph.D. thesis, University of Sevilla, Sevilla, Spain, 1981). However, as in other *Rhizobium* species, the most efficient plasmid with chromosome-mobilizing ability is R68.45 (15; Megías, Ph.D. thesis, 1981).

Usual frequencies of mobilization of chromosomal markers by R68.45 are higher than reversion rates. For instance, all the auxotrophic mutations reported in this paper had reversion frequencies lower than 10^{-6}, whereas the chance of detecting single-marker transfer in an R68.45-mediated cross was 10^{-4} to 10^{-5} per donor cell. Thus, accurate measures of transfer frequencies may be readily obtained. Frequency ranges described in this paper are similar to those reported for *R. meliloti* (10, 21) and for the original host of R68.45, *P. aeruginosa* (13).

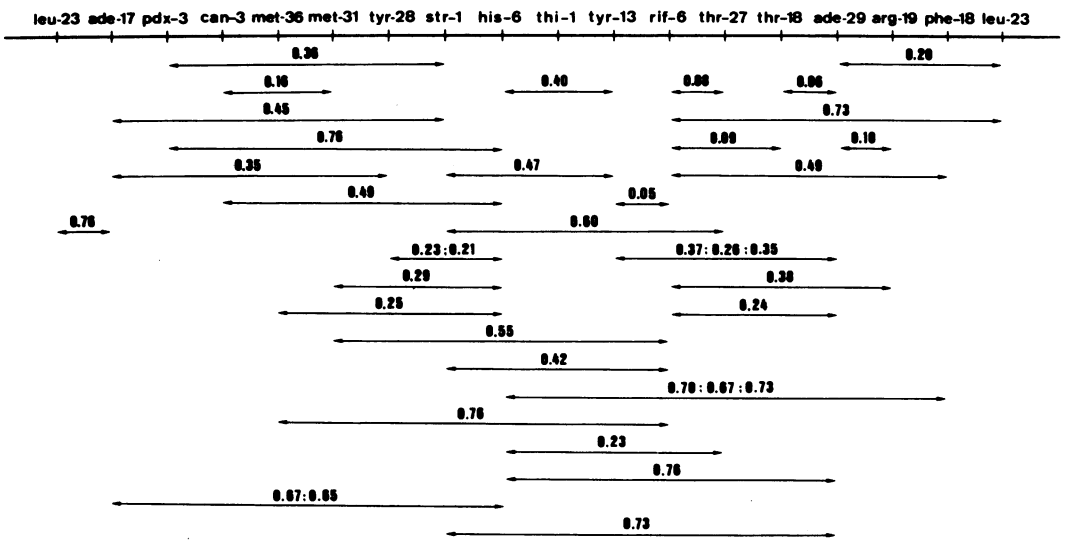

FIG. 1. Preliminary linkage map of the *R. trifolii* RS176 chromosome. Numbers beside gene symbols indicate allele numbers. Numbers on the arrows indicate conjugal linkage percentages.

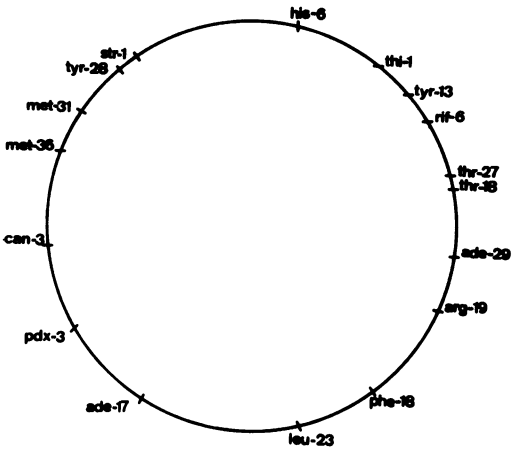

FIG. 2. Circular linkage map of the *R. trifolii* RS176 chromosome. Distances are about inversely proportional to the linkage frequencies. Minimal linkage values (3%) would represent, at least, one-fourth of the total length of the chromosome.

Another advantage of using plasmid R68.45 for mapping purposes in *Rhizobium* spp. is the large size of chromosomal fragments mobilized by the plasmid. Subsequently, simultaneous transfer of distant markers may be readily detected in appropriate crosses. For instance, marker pairs showing low linkage frequencies, such as *his-6/phe-18*, *met-36/rif-6*, and *his-6/pdx-3*, may be located as far apart as one-fourth of the total length of the chromosome. These results agree with those reported for other *Rhizobium* species (4, 10, 21).

Transformation of linkage measures into additive map distances is easily obtained by use of formulas such as that of Wu (28) or that of Kemper (19). These equations were first proposed for use in cotransduction experiments, but they are also suitable for conjugal mapping (21; Megías, Ph.D. Thesis, 1981). We have chosen to use Kemper's equation because it does not require any assessment of the length of transferred chromosomal segments.

Unlike *R. meliloti* (10, 11, 25) and *R. leguminosarum* (8) general transduction systems have not yet been described for *R. trifolii*. Thus, conjugal mapping mediated by plasmid R68.45 is the only method now available for extensive mapping studies in *R. trifolii*. We hope it will be suitable for further genetic analysis of nitrogen-fixing clover symbiosis.

ACKNOWLEDGMENTS

We are grateful to John Beringer for providing plasmid R68.45 and to Josep Casadesús for helpful suggestions during the preparation of the manuscript.

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