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Genetics

Genetic mapping of *Rhizobium meliloti*

(R factor RP4/chromosomal mobilization/nitrogen fixation)

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ABSTRACT **The drug resistance factor RP4, originally isolated in *Pseudomonas*, was transferred to *Rhizobium meliloti*. In that strain, RP4 promotes conjugational transfer of chromosomal markers to form haploid recombinants. This mating system has been used to construct a linkage map of *R. meliloti*.**

Rhizobium are important because they fix atmospheric nitrogen in the root nodules of legumes. The bacteria have been studied for many years, but thus far no system has been available for convenient and extensive genetic analysis.

We describe here a genetic system for *Rhizobium meliloti* based on conjugation. During mating the drug resistance factor RP4 can mobilize the bacterial chromosome to produce ultimately recombinant progeny. We have used this system to construct a linkage map of *R. meliloti*. This approach should also be suitable for developing genetics in other bacteria where it cannot yet be done.

A number of reports have described genetic transfer in *Rhizobium*. As early as 1953 R. Balassa (cited by G. Balassa in ref. 1) reported transformation among three different species, and there have been occasional reports since then (2–5), but only recently has linkage been shown (6). Sik and Orosz (7) have reported one case of special transduction. Kowalski (8) found general transduction, but although many different markers could be transduced there was only one example of cotransduction (9).

There have been several reports of conjugation. Higashi (10) reported transfer of nodulation specificity between two species of *Rhizobium* and Lorkiewicz *et al.* (11) described transfer of auxotrophic markers in *R. trifolii*. We are not aware of subsequent reports in either case. Heumann (12) reported mating and a circular linkage map in a strain said to be *R. lupini*. Unfortunately this is difficult to evaluate because the strain no longer nodulates any legume (13) and, unlike most *Rhizobium*, produces colored colonies on agar. Because one of our goals is a genetic analysis of the symbiotic process, we found it necessary to develop a workable genetic system in an effective nodulating strain of *Rhizobium*.

RP4 is a P-type drug resistance factor that confers resistance to carbenicillin, tetracycline, and kanamycin/neomycin. It was found in a clinical *Pseudomonas aeruginosa* isolate that had become resistant to carbenicillin (14). Datta *et al.* (15) showed that RP4 will mate into most Gram-negative bacteria, including *Rhizobium*. Since Stanisich and Holloway (16) showed that P-type R factors can mobilize the *Pseudomonas* chromosome, we were prompted to try RP4 in *Rhizobium*. Towner and Vivian (17) have reported RP4 promoted mobilization and linkage in Acinetobacter, yet Jacob *et al.* (18) found only marginal mobilization by RP4 in *R. leguminosarum*. However, since our work was done Beringer and Hopwood (19) reported chromosome mobilization and linkage in *R. leguminosarum* with another P-type R factor, R68-45.

We have presented preliminary reports on this work (20, 21). The results reported here, together with those of others (19), indicate that it is now possible to do extensive genetics of nodulating *Rhizobium* strains.

MATERIALS AND METHODS

Strains. The parental strain Rm2011 is *R. meliloti* 2011 *str3*, a streptomycin-resistant mutant isolated by J. Denarie (C.N.R.A., Versailles) from *R. meliloti*, SU47 (P. S. Nutman, Rothamsted Experimental Station). It has a partial requirement for biotin and forms effective nodules on alfalfa.

Escherichia coli J53 *pro*$^-$ *met*$^-$/RP4 was obtained from G. Jacoby. It was mated with Rm2011; the recipients were selected with tetracycline (10 μg/ml) and counterselection with streptomycin (250 μg/ml) to yield Rm2011/RP4. Other *R. meliloti*/RP4 strains were derived by mating with one or the other of these.

Media. Unlike most rhizobia, Rm2011 grows well on standard *E. coli* media. Rich medium is LB broth (22). Synthetic medium is M9 (22) supplemented with 0.2% sucrose, 0.5 μg of biotin per ml, and 20 μg of amino acids or bases per ml when needed. Bacteria are routinely grown in LB unless otherwise stated. The nodulation assay is done as described by Vincent (23).

Drug-resistant mutants are isolated on supplemented LB plates. *R. meliloti*/RP4 are resistant to carbenicillin (50 μg/ml), tetracycline (10 μg/ml), and neomycin (50 μg/ml). RP4 transfer is selected with tetracycline.

Ethylmethylsulfonate Mutagenesis. A stationary phase culture was washed twice and resuspended in half its original volume of Tris/saline at pH 7.6 (22). Two drops of ethylmethylsulfonate were added per ml and the mixture was incubated 1 hr at 30°. Cells were then washed with 20 mM $Na_2S_2O_3$, diluted 10-fold into LB, and grown overnight for phenotypic expression. This procedure gives 25% survival and a 300-fold increase in rifampin-resistant colonies.

Auxotrophs. Enrichment was a modification of the penicillin method (24). Mutagenized cells were grown to logarithmic phase in LB, washed twice, resuspended in supplemented M9 medium and starved for 5 hr. Carbenicillin (Pfizer) was added at 5 mg/ml and the culture was shaken at 30° for 4 days. The cells were washed to remove the drug and plated on LB. Colonies appearing after 3 days were replicated onto supplemented M9 plates to identify auxotrophs, which were then characterized by streaking onto minimal plates supplemented with growth factor pools described by Holliday (25). This procedure gave 10^{-4} survival and 2–5% auxotrophs among the survivors. Multiply marked strains were isolated by successive mutagenesis and enrichment.

Liquid Mating. Equal volumes of logarithmic phase R$^+$ donor and stationary phase recipient cultures were mixed and incubated without shaking for 3 h at 34°, then diluted and plated on appropriate selective media.

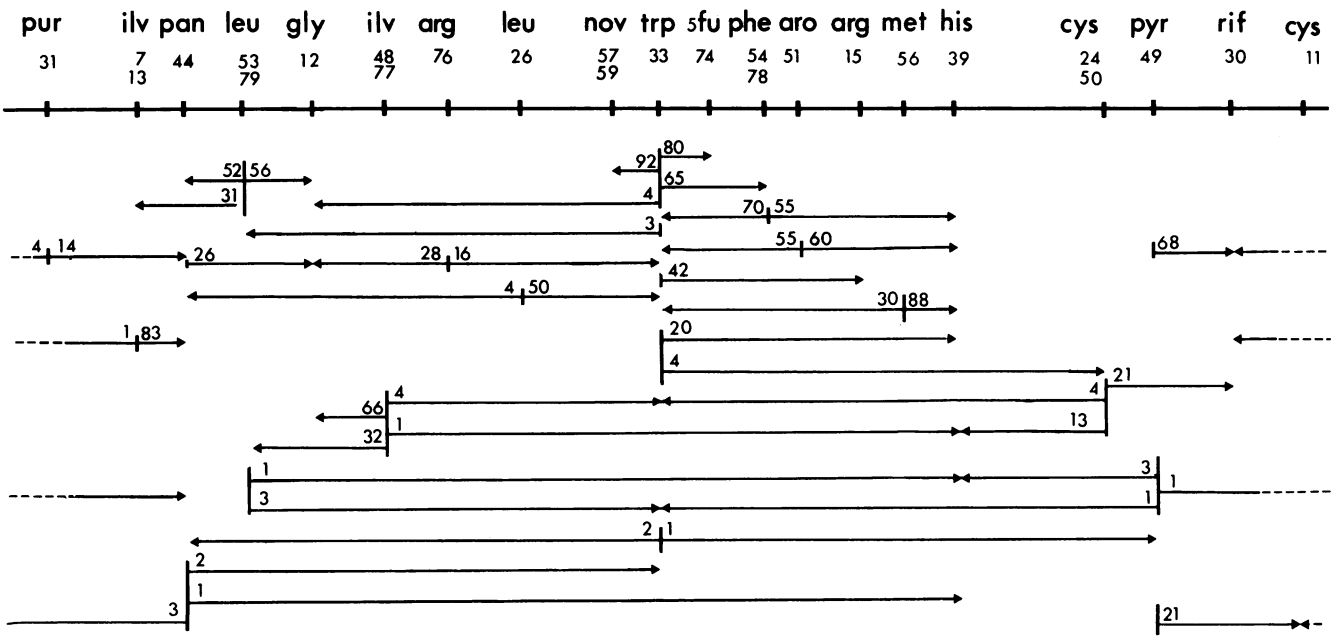

FIG. 1. Abbreviations: rif, rifampin (Calbiochem) resistance to 100 μg/ml; nov, novobiocin (Sigma) resistance to 50 μg/ml; 5fu, 5-fluorouracil (Calbiochem) resistance to 100 μg/ml. Isolate numbers are listed immediately below the markers. Each arrow represents the percent linkage averaged over 1–5 crosses, with the selected marker at the tail and the unselected marker at the head. Linkage less than 0.5% was found between: *pyr49-gly12, leu53-rif30, ilv13-his39, pur31-trp33, his39,* and *trp33-ilv13.*

Plate Matings. One-half milliliter of logarithmic phase R^+ donor was mixed with 0.5 ml of stationary phase recipient culture. The mixture was spread on a warmed LB plate and incubated overnight at 34°. Ten milliliters of saline was added to the lawn, which was then scraped into a centrifuge tube, washed twice, resuspended in 5 ml of saline, diluted, and plated. Each parent alone was carried through the same procedure as a control.

Linkage frequencies represent the scoring of 100 to 200 colonies for each unselected marker in each experiment.

RESULTS

We chose *R. meliloti* because of its relatively fast growth on standard *E. coli* media (2½ hr doubling time in LB), because auxotrophs had already been isolated in this strain (26), and because the alfalfa seedlings it nodulates are small and easily handled.

In liquid matings RP4 was transferred between *R. meliloti* at 10^{-4} per donor cell. With a nonreverting *trp*$^-$ recipient we have found no *trp*$^+$ transfer ($<10^{-9}$ per donor) in liquid. The plate mating technique increased RP4 transfer to 10^{-1} per donor, and colonies with recombinant phenotype were found at a frequency of 10^{-5} to 10^{-6} per donor.

These colonies appeared to represent true haploid chromosomal recombinants by several criteria: (*a*) when purified, colonies were stable and did not segregate parental types; (*b*) unselected donor markers were found among the selected colonies at characteristic and reproducible linkage frequencies; and (*c*) the unselected markers may be auxotrophic and therefore presumably recessive.

We have done a large number of matings with pairs of strains carrying various combinations of markers. (All multiply marked strains were constructed by successive mutagenesis rather than by mating, so that all the results are from virgin mating partners.) Not all marker pairs show linkage (see legend to Fig. 1). For pairs that do, the linkage frequencies are summarized in Fig. 1. There is a single linkage group with no ends, which can be represented as the circle in Fig. 2.

DISCUSSION

Our results clearly demonstrate transfer between *R. meliloti* strains of genetic markers that are presumably located on the bacterial chromosome. The exconjugants with recombinant phenotype appear to be haploids resulting from true crossing-over, since they do not segregate and since recessive markers may be introduced from the donor. The crosses give an empirical linkage map that is linear. Selection of a pair of donor markers implies in most cases inheritance of a marker located between them on the map: for example, in the cross *arg*$^-$ × *trp*$^-$ *his*$^-$, simultaneous selection for *trp*$^+$ and *his*$^+$ gives recombinants of which 98% are *arg*$^-$ (compare Fig. 1).

We have as yet no information about the mechanism of transfer. Since for different marker pairs we find a broad continuum of linkage frequencies ranging from 0.5 to 92%, there is no reason to suppose that more than one mechanism is involved. Transfer frequencies for all markers are roughly the

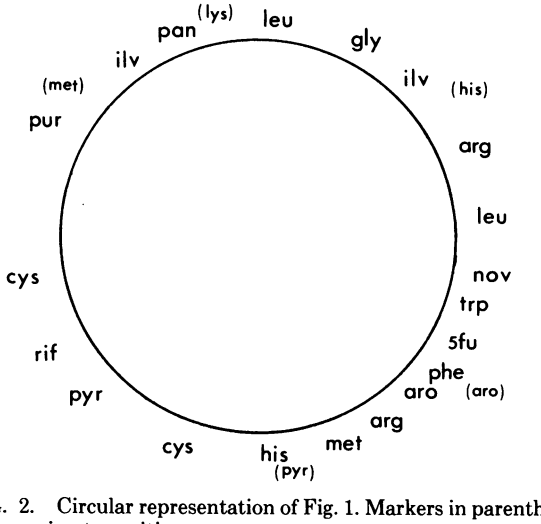

FIG. 2. Circular representation of Fig. 1. Markers in parentheses are approximate positions.

same and give no indication of a polarity of transfer. This is similar to $F^+ \times F^-$ matings in *E. coli*, where the origin and polarity of transfer that are characteristic of a single mating event are masked in the overall population by the large number of such events with independent origins that take place simultaneously. This is likely to be the case here too. We have in fact one *R. meliloti* derivative, which appeared spontaneously, that gives a higher (10 to 100 ×) frequency of transfer, apparently with both an origin and polarity, like an *E. coli* Hfr male. Thus, at this early stage of our understanding, the *E. coli* F system seems to be a useful model for RP4-mediated transfer in *R. meliloti*

F-type Hfr bacteria can be isolated selectively through the use of an F_{ts} derivative that is thermosensitive for episomal replication (27). We have isolated an analogous $RP4_{ts}$.

We expect this mating system to be of great value in the analysis of *Rhizobium* genetics and physiology and in the isolation of strains with useful combinations of markers. The most interesting properties of *Rhizobium*, namely, nodulation and nitrogen-fixation, cannot easily be selected in free-living bacteria. The mating system will allow the relevant genes to be manipulated by their linkage to more easily handled markers.

Finally, from a general point of view our results are encouraging for the development of genetics in bacterial strains where no genetic system is yet known.

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