JOURNAL OF BACTERIOLOGY, Jan. 1992, p. 324–326
0021-9193/92/010324-03$02.00/0

Vol. 174, No. 1

A Directional, High-Frequency Chromosomal Mobilization System for Genetic Mapping of *Rhizobium meliloti*

SHOSHANA KLEIN,[1] KARIN LOHMAN,[2] RALPH CLOVER,[3] GRAHAM C. WALKER,[4]* AND ETHAN R. SIGNER[4]

Biology Department, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139[4]; Department of Genetics, Institute of Life Sciences, The Hebrew University, Jerusalem, Israel[1]; Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706[2]; and Department of Biology, Northeastern University, Boston, Massachusetts 02115[3]

Received 21 May 1991/Accepted 2 November 1991

A system for mapping of the *Rhizobium meliloti* chromosome that utilizes transposon Tn*5*-Mob, which carries the mobilization site of IncP plasmid RP4 (R. Simon, Mol. Gen. Genet. 196:413–420, 1984), was developed. Insertions of Tn*5*-Mob that were located at particular sites on the *R. meliloti* chromosome were isolated and served as origins of high-frequency chromosomal transfer when IncP *tra* functions were provided in *trans*. This approach is, in principle, applicable to any gram-negative bacterium in which Tn*5* can transpose and into which IncP plasmids can conjugate.

The gram-negative bacterium *Rhizobium meliloti* has been the object of considerable genetic analysis because of its ability to form a symbiotic association with alfalfa that results in formation of nitrogen-fixing nodules. The *R. meliloti* genome is complex and consists of a circular chromosome and two megaplasmids of ca. 1,500 kb each; in the SU47/Rm1021 strain background, these have been designated pRmeSU47a and pRmeSU47b (1, 7). In recent years, a great deal of attention has been focused on the two megaplasmids since they carry many of the genes required for symbiosis. For example, pRmeSU47a carries the *nod* genes required for nodule induction and the *nif* and *fix* genes which are required for nitrogen fixation (1) while pRmeSU47b carries genes required for exopolysaccharide biosynthesis, dicarboxylic acid transport, and bacteroid development (3). Both genetic and physical maps of the regions of pRmeSU47a carrying the *nod*, *nif*, and *fix* loci have been published (2, 9, 19). A circular linkage map of pRmeSU47b has recently been prepared (3), as have genetic and physical maps of the regions of the megaplasmid required for exopolysaccharide biosynthesis (8, 12).

In earlier work, genetic maps of the *R. meliloti* chromosome itself were prepared (10, 11, 16). These studies utilized IncP plasmid RP4 (and its derivatives), a conjugal plasmid that can mobilize chromosomal segments in a manner conceptually analogous to mobilization of the *Escherichia coli* chromosome by the F plasmid (17). By mating an RP4-carrying strain with a suitable recipient and selecting for recombination of a mobilized chromosomal fragment into the genome of the recipient, genetic loci can be mapped with respect to one another. However, this RP4-based system has two major drawbacks. (i) Mobilization of any given locus occurs at low frequency (10^{-6} to 10^{-8}). (ii) Mobilization occurs from random (or nearly random) sites with equal frequency of transfer of markers equidistant from any given point in either direction.

We therefore devised a system for genetic mapping of the *R. meliloti* chromosome that utilizes Tn*5*-Mob, a derivative of Tn*5* constructed by Simon (18) that carries the origin of transfer (*oriT*) of plasmid RP4. Simon (18) had previously shown that introduction of Tn*5*-Mob into the chromosome of *E. coli* resulted in directional mobilization when IncP *tra* (conjugal transfer) functions were supplied in *trans* and that the *R. meliloti* plasmids could also be mobilized by use of Tn*5*-Mob. In addition, Yakobson and Guiney (20) had previously shown that a related Tn*5* derivative, Tn*5-11*, which carries the *oriT* gene of an IncP plasmid, can be used to mobilize the chromosomes of *E. coli* and *R. meliloti* in a polarized manner. We have previously taken advantage of Tn*5-11* (7) to map various symbiotic loci to either pRmeSU47a or pRmeSU47b (7, 8, 12). We therefore constructed a series of *R. meliloti* SU47 derivatives with Tn*5*-Mob inserted near known chromosomal loci in both possible orientations (Table 1). We show here that the approximate

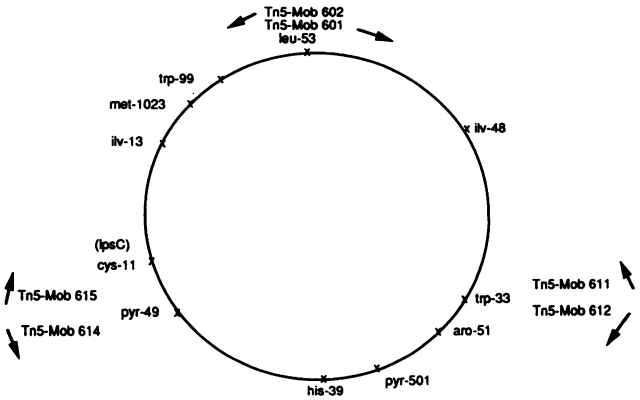

FIG. 1. Linkage map of the *R. meliloti* genome, showing the relative positions of several auxotrophic markers, *lpsC*, and the Tn*5*-Mob insertions. *lpsC* is shown in parentheses because its position relative to that of *cys-11* was not determined. Chromosomal mobilization is initiated within the Tn*5* sequences and proceeds in the directions indicated by the arrows. Thus, *trp-99* is transferred early from Tn*5*-Mob insertion Ω601, while *ilv-48* is transferred early from Tn*5*-Mob insertion Ω602.

* Corresponding author.

TABLE 1. Bacterial strains

Strain name	Relevant characteristics	Source
Rm1021	SU47 *str-21*	F. Ausubel
Rm3357	SU47 *str-3 spc-1 rif-1 nov-57 trp-33 his-39 leu-53*	H. Meade
Rm3359	SU47 *str-3 spc-1 rif-1 nov-57 trp-33 his-39 pyr-49*	H. Meade
Rm3356	SU47 *str-3 spc-1 rif-1 his-39 met-56*	H. Meade
Rm3348	SU47 *str-3 spc-1 rif-1 his-39 ilv-48 trp-33*	H. Meade
Rm2013	SU47 *str-3 ilv-13*	H. Meade
Rm5050	SU47 *str-21 met-1025*::Tn*5*	S. Long
Rm6826	SU47 *str-3 spc-1 rif-1 his-39 trp-33 aro-51 pyr-501*::Tn*5*	This work
Rm6827	SU47 *str-3 pyr-501*::Tn*5 cys-11*	This work
Rm6661	SU47 *str-3 spc-1 rif-1 nov-57 his-39 trp-33* Ω601::Tn*5*-Mob	This work
Rm6662	SU47 *str-3 spc-1 rif-1 nov-57 his-39 trp-33* Ω602::Tn*5*-Mob	This work
Rm6672	SU47 *str-21* Ω601::Tn*5*-Mob	This work
Rm6673	SU47 *str-21* Ω602::Tn*5*-Mob	This work
Rm6761	SU47 *str-21* Ω611::Tn*5*-Mob	This work
Rm6762	SU47 *str-21* Ω612::Tn*5*-Mob	This work
Rm6847	SU47 *rif-100* Ω614::Tn*5*-Mob	This work
Rm6849	SU47 *rif-100* Ω615::Tn*5*-Mob	This work
Rm6679	Rm3357 Ω601::Tn*5*-Mob	This work
Rm6680	Rm3357 Ω602::Tn*5*-Mob	This work
Rm6755	Rm3359 *trp*$^+$ Ω611::Tn*5*-Mob	This work
Rm6753	Rm3359 Ω612::Tn*5*-Mob	This work
Rm6738	Rm3359 Ω614::Tn*5*-Mob	This work
Rm6730	Rm3359 Ω615::Tn*5*-Mob	This work

location of any chromosomal marker can be determined on the basis of the frequency of its transfer from strains carrying these Tn*5*-Mob insertions.

Tn*5*-Mob was linked to previously mapped loci on the *R. meliloti* chromosome by the following procedure. Derivatives of *R. meliloti* Rm1021 (RmSU47 *str-21*) that carried random insertions of Tn*5*-Mob were obtained by mating Rm1021 with a derivative of *E. coli* S17 which carried pSUP5011 (the plasmid used to deliver Tn*5*-Mob) (18), selected for Tn*5*-Mob-encoded neomycin (Nm; 200 μg/ml) resistance, and counterselected with streptomycin (200 μg/ml). Approximately 10,000 Rm1021 derivatives containing random Tn*5*-Mob insertions were then pooled, and a lysate of bacteriophage φM12 (6) was prepared. This lysate was used to transduce the random Tn*5*-Mob insertions into a number of multiple auxotrophic strains, including Rm3359 (SU47 *str-3 trp-33 his-39 pyr-49 nov-57 spc-1 rif-1*) (16) and Rm3357 (*str-3 trp-33 his-39 leu-53 nov-57 spc-1 rif-1*) (16). Selection was for Nm resistance plus prototrophy for individual nutrients, namely, leucine (to give insertions linked by transduction to *leu-53*), tryptophan (to give insertions linked to *trp-33*), or pyrimidine (to give insertions linked to *pyr-49*) on M9 medium (13) containing Nm and appropriate supplements. The various Tn*5*-Mob insertions were later moved into other genetic backgrounds, as necessary, by φM12-mediated transduction with selection for Nm resistance.

For each of the three auxotrophic loci listed above, we chose up to 10 isolates with cotransducible Tn*5*-Mob insertions. These strains were then tested as donors in triparental matings using suitable auxotrophs as recipients (see Table 2 and Fig. 1). The third parent was an *E. coli* strain carrying self-transmissible plasmid pRK600 (7), which provides the

TABLE 2. Transfer of auxotrophy markers from Tn*5*-Mob inserts

Insert	Locus used for selection by linkage	Donor	Recipient	Marker selected for prototrophy	Frequency of transfer[a]
Ω601	*leu-53*	Rm6672	Rm3348	*ilv-48*	$<10^{-8}$
Ω601	*leu-53*	Rm6672	Rm3348	*trp-33*	$<10^{-8}$
Ω601	*leu-53*	Rm6661	Rm5050	*met-1023*	8.0×10^{-5}
Ω601	*leu-53*	Rm6661	Rm2013	*ilv-13*	1.0×10^{-4}
Ω601	*leu-53*	Rm6661	Rm6827	*cys-11*	2.0×10^{-6}
Ω602	*leu-53*	Rm6673	Rm3348	*ilv-48*	4.7×10^{-4}
Ω602	*leu-53*	Rm6673	Rm3348	*trp-33*	2.8×10^{-4}
Ω602	*leu-53*	Rm6662	Rm5050	*met-1023*	$<10^{-7}$
Ω602	*leu-53*	Rm6662	Rm2013	*ilv-13*	$<10^{-7}$
Ω602	*leu-53*	Rm6662	Rm6827	*cys-11*	$<10^{-7}$
Ω611	*trp-33*	Rm6761	Rm6826	*aro-51*	9.0×10^{-4}
Ω611	*trp-33*	Rm6761	Rm6826	*pyr-501*	3.2×10^{-4}
Ω612	*trp-33*	Rm6762	Rm6826	*aro-51*	$<10^{-7}$
Ω612	*trp-33*	Rm6762	Rm6826	*pyr-501*	$<10^{-7}$
Ω614	*pyr-49*	Rm6847	Rm6827	*pyr-501*	$<10^{-8}$
Ω614	*pyr-49*	Rm6847	Rm6827	*cys-11*	1.4×10^{-4}
Ω615	*pyr-49*	Rm6849	Rm6827	*pyr-501*	3.2×10^{-5}
Ω615	*pyr-49*	Rm6849	Rm6827	*cys-11*	$<10^{-8}$

[a] Frequency is expressed relative to total recipient cells.

IncP *tra* functions needed for mobilization of DNA carrying Tn*5*-Mob. pRK600 can conjugally transfer itself to *R. meliloti* but cannot replicate in *R. meliloti*. We looked for transfer of markers that were known by previous mapping studies utilizing RP4 (16) to be linked to the region in which Tn*5*-Mob was inserted. Selection and counterselection employed appropriate auxotrophies and/or resistance to rifampin (50 μg/ml) or streptomycin. We found that transfer and recombination of markers occurred at frequencies of up to 10^{-3} and that the frequency of rescue of any locus depended on its distance from the origin of transfer (i.e., from the Tn*5*-Mob insertion in the donor). Moreover, mobilization of chromosomal segments was directional: for any given Tn*5*-Mob insertion, high frequency of recombination occurred only for markers on one side of that insertion. For instance, Tn*5*-Mob insertions Ω601 and Ω602 are both linked by transduction to *leu-53*. From a donor strain carrying Tn*5*-Mob insertion Ω601, transfer of loci *met-1023* and *ilv-13* (both counterclockwise to *leu-53* in Fig. 1) occurs at frequencies of 8×10^{-5} and 1×10^{-4}, respectively, whereas transfer of *ilv-48* and *trp-33* (clockwise to *leu-53* in Fig. 1) is at very low frequencies. On the other hand, from a donor strain carrying Tn*5*-Mob insertion Ω602, transfer of loci *met-1023* and *ilv-13* occurs at very low frequencies, whereas transfer of *ilv-48* and *trp-33* occurs at frequencies of 4.7×10^{-4} and 2.8×10^{-4}, respectively (Table 2). Since Tn*5*-Mob inserted in either orientation with approximately equal frequencies during construction of the original Tn*5*-Mob library, it was possible to choose insertions linked to each of the three loci, *leu-53*, *trp-33*, and *pyr-49*, which served as origins of transfer in either direction, and these are listed in Table 2. With certain exceptions (discussed below), any chromosomal locus can be mobilized at high frequency from at least one of these six origins of transfer, which divide the chromosome into three large, easily mobilizable, overlapping segments.

We have used this system to find the approximate location of the *lpsC* locus on the *R. meliloti* chromosome (4). *lpsC27-1*::Tn*5-233* was transduced into each of six donor strains, which were then mated with Rm1021 (RmSU47 *str-21*) (Table 3). It was not necessary to determine frequen-

TABLE 3. Mapping of *lpsC*[a]

Insert	Donor	Counter-selection	No. of colonies
Ω601	Rm6679 *lpsC 27-1*::Tn*5-233*	*trp*, *his*	1,450
Ω602	Rm6680 *lpsC 27-1*::Tn*5-233*	*his*	320
Ω611	Rm6755 *lpsC 27-1*::Tn*5-233*	*pyr*	400
Ω612	Rm6753 *lpsC 27-1*::Tn*5-233*	*his*, *pyr*	400
Ω614	Rm6738 *lpsC 27-1*::Tn*5-233*	*trp*, *his*	>4,000
Ω615	Rm6730 *lpsC 27-1*::Tn*5-233*	*trp*	346

[a] Matings were performed overnight on LB agar (13) at 30°C. Mating mixtures were resuspended and washed twice in saline. Undiluted suspensions were plated on M9 agar supplemented with gentamicin (30 μg/ml) and spectinomycin (150 μg/ml) to select for transfer of Tn*5-233* (5), as well as amino acids or uracil, as appropriate. Control plates with donors, a helper, and a recipient (Rm1021) had no colonies, with the exception of Rm6680 *lpsC 27-1*::Tn*5-233*, which gave 12 colonies on selective medium.

cies of transfer, since donors carrying insertions Ω614 and Ω601 clearly gave more colonies than any other donors. (It is interesting that every mating gave at least some colonies, suggesting that in a long, overnight mating it may be possible to transfer the entire chromosome from a Tn*5*-Mob insertion). Subsequent transductional mapping indicated that *lpsC* is 52% linked to *cys-11*, thereby confirming that *lpsC* is in the sector between Ω601 and Ω614.

Some markers which are tightly linked to the nearest Tn*5*-Mob insertion might fail to be transferred at high frequency from any origin of transfer. This could be for one of two reasons: (i) the marker lies between two Tn*5*-Mob insertions, each of which transfers away from the direction of the marker, or (ii) the marker is so tightly linked to the Tn*5*-Mob insertion that, following transfer, little recombination can occur between the origin of transfer and the marker, such that recombinational rescue of the marker in question is not detected. In any event, such markers should be linked by transduction to one pair of Tn*5*-Mob insertions.

The approach we have described here is, in principle, applicable to any gram-negative bacterium in which Tn*5* can transpose and into which IncP plasmids can conjugate. An alternative method for obtaining Tn*5*-Mob insertions in opposite orientations at a given point without the use of a transducing bacteriophage would be first to obtain an insertion of an Nm-sensitive derivative of Tn*5*, for example, Tn*5-233* (5), and then to substitute Tn*5*-Mob for the original Tn*5* derivative by recombination between the inverted repeats of the two Tn*5* derivatives as we have previously described (5). Tn*5*-Mob would be expected to recombine into the genome in both orientations at the point of insertion of Tn*5-233*.

This work was supported by Public Health Service grant GM31030 to E.R.S. and G.C.W. K.L. was supported in part by the Undergraduate Research Opportunities Program at the Massachusetts Institute of Technology.

REFERENCES

1. **Banfalvi, Z., V. Sakanyan, C. Koncz, A. Kiss, I. Dusha, and A. Kondorosi.** 1981. Location of nodulation and nitrogen fixation genes on a high molecular weight plasmid of *R. meliloti*. Mol. Gen. Genet. **184**:318–325.
2. **Batut, J., B. Terzaghi, M. Gherardi, M. Huguet, E. Terzaghi, A. M. Garnerone, P. Boistard, and T. Huguet.** 1985. Localization of a symbiotic *fix* region on *Rhizobium meliloti* pSym megaplasmid more than 200 kilobases from the *nod-nif* region. Mol. Gen. Genet. **199**:232–239.
3. **Charles, T. C., and T. F. Finan.** 1990. Genetic map of *Rhizobium meliloti* megaplasmid pRmeSU47b. J. Bacteriol. **172**:2469–2476.
4. **Clover, R. H., J. Keiber, and E. R. Signer.** 1989. Lipopolysaccharide mutants of *R. meliloti* are not defective in symbiosis. J. Bacteriol. **171**:3961–3967.
5. **DeVos, G. F., G. C. Walker, and E. R. Signer.** 1986. Genetic manipulation in *Rhizobium meliloti* using two new transposon Tn*5* derivatives. Mol. Gen. Genet. **204**:485–491.
6. **Finan, T. M., E. Hartweig, K. LeMieux, K. Bergman, G. C. Walker, and E. R. Signer.** 1984. General transduction in *Rhizobium meliloti*. J. Bacteriol. **159**:120–124.
7. **Finan, T. M., B. Kunkel, G. F. De Vos, and E. R. Signer.** 1986. Second symbiotic megaplasmid in *R. meliloti* carrying exopolysaccharide and thiamine synthesis genes. J. Bacteriol. **167**: 66–72.
8. **Glazebrook, Jr., and G. C. Walker.** 1989. A novel exopolysaccharide can function in place of the Calcofluor-binding exopolysaccharide in nodulation of alfalfa by *Rhizobium meliloti*. Cell **56**:661–672.
9. **Julliot, J. S., I. Dusha, M. H. Renalier, B. Terzaghi, A. M. Garnerone, and P. Boistard.** 1984. An RP4-prime containing a 285kb fragment of *Rhizobium meliloti* pSym megaplasmid: structural characterization and utilization for genetic studies of symbiotic functions controlled by pSym. Mol. Gen. Genet. **193**:17–26.
10. **Kondorosi, A., G. B. Kiss, T. Forrai, E. Vincze, and Z. Banfalvi.** 1977. Circular linkage map of *Rhizobium meliloti* chromosome. Nature (London) **268**:525–527.
11. **Kondorosi, A., E. Vincze, A. W. B. Johnston, and J. E. Beringer.** 1980. A comparison of three *Rhizobium* linkage maps. Mol. Gen. Genet. **178**:403–408.
12. **Long, S., J. W. Reed, J. W. Himawan, and G. C. Walker.** 1988. Genetic analysis of a cluster of genes required for synthesis of the Calcofluor-binding exopolysaccharide of *Rhizobium meliloti*. J. Bacteriol. **170**:4239–4248.
13. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. **Martin, M. O., and S. R. Long.** 1984. Generalized transduction in *Rhizobium meliloti*. J. Bacteriol. **159**:125–129.
15. **Meade, H. M.** 1977. Ph.D. thesis. Massachusetts Institute of Technology, Cambridge.
16. **Meade, H. M., and E. R. Signer.** 1977. Genetic mapping of *R. meliloti*. Proc. Natl. Acad. Sci. USA **74**:2076–2078.
17. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Simon, R.** 1984. High frequency mobilization of gram-negative bacterial replicons by the in vitro constructed Tn*5*-Mob transposon. Mol. Gen. Genet. **196**:413–420.
19. **Swanson, J. A., J. K. Tu, J. Ogawa, R. Sanga, R. F. Fisher, and S. R. Long.** 1987. Extended region of nodulation genes in *R. meliloti* 1021. Genetics **117**:181–189.
20. **Yakobson, E. A., and D. G. Guiney, Jr.** 1984. Conjugal transfer of bacterial chromosomes mediated by the RK2 plasmid transfer origin cloned into transposon Tn*5*. J. Bacteriol. **160**:451–453.