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Genetic Mapping of Symbiotic Loci on the *Rhizobium meliloti* Chromosome

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To facilitate genetic analyses of *Rhizobium meliloti* genes that are involved in symbiosis, we determined the map positions of 11 symbiotic loci on the *R. meliloti* chromosome by using a combination of the Tn*5*-Mob conjugational transfer method described by Klein *et al.* (S. Klein, K. Lohmann, G. C. Walker, and E. R. Signer. J. Bacteriol. 174:324-326, 1992) and co-transduction of genetic markers by bacteriophage ΦM12. Loci involved in effective nodule formation (*fix-379*, *fix-382*, *fix-383*, *fix-385*, and *fix-388*), polysaccharide synthesis (*exoR*, *exoS*, *exoC*, and *ndvB*), nodule invasion (*exoD*), and nitrogen regulation (*ntrA*) were ordered with respect to previously mapped markers and each other. The positions of two other loci, *degP* and *pho-1*, were also determined.

The gram-negative bacterium *Rhizobium meliloti* Dangeard fixes atmospheric nitrogen as an endosymbiont dwelling in nodules on alfalfa roots. Its genome consists of a chromosome of approximately 4,000 kb and two large megaplasmids of approximately 1,500 kb each, designated pSymA and pSymB (Banfalvi *et al.* 1981; Finan *et al.* 1986). These megaplasmids have been the subjects of intensive study, because many genes involved in symbiosis are located on them. pSymA carries the *nod* and *hsn* genes, which are required for initiation of symbiosis, as well as the *nif* and *fix* genes, which are needed for nitrogen fixation (Banfalvi *et al.* 1981). pSymB also carries genes involved in exopolysaccharide synthesis, dicarboxylate transport, and bacteroid development, all of which are required for effective symbiosis (Charles and Finan 1990). Extensive genetic and physical maps of the regions of pSymA that contain the *nod*, *nif*, and *fix* loci are available (Batut *et al.* 1985; Juillot *et al.* 1984; Swanson *et al.* 1987). Charles and Finan have recently constructed a circular linkage map of pSymB by using a set of transposon insertions that are co-transducible by bacteriophage ΦM12 (Charles and Finan 1990). Physical maps of the *exo* and *exp* regions of pSymB have been prepared (Glazebrook and Walker 1989; Long *et al.* 1988).

In addition, many genes required for effective symbiosis are located on the chromosome. However, detailed genetic maps of the *R. meliloti* chromosome have not been developed, and few map positions of symbiotic loci have been reported. Klein *et al.* (1992) developed a system for conjugational mapping of *R. meliloti* genes by using the transposon Tn*5*-Mob (Simon 1984), which contains the origin of transfer of the broad host range plasmid RP4. Consequently, *R. meliloti* chromosomes that contain insertions of this transposon can be mobilized into other strains when transfer functions are supplied in *trans*. A set of strains containing insertions of Tn*5*-Mob at several defined locations around the chromosome was constructed, and markers of interest were mapped by determining which Tn*5*-Mob insertion strains transferred them at the highest frequencies (Klein *et al.* 1992). This system was tested by using it to map some of the auxotrophic mutations localized previously by Meade and Signer (1977), several other auxotrophic mutations, and the *lpsB* locus (Klein *et al.* 1992).

We have used this set of Tn*5*-Mob insertion strains to map symbiotic loci on the *R. meliloti* chromosome. These include a set of Tn*phoA* insertion mutations that cause induction of Fix$^-$ nodules on alfalfa (*fix-379*, *fix-382*, *fix-383*, *fix-385*, and *fix-388*; Long *et al.* 1988) and loci involved in polysaccharide synthesis (*exoR* and *exoS*, Doherty *et al.* 1988; *exoC*, Leigh *et al.* 1985; and *ndvB*, Dylan *et al.* 1986 and Geremia *et al.* 1987), nodule invasion (*exoD*, Reed and Walker 1991a,b), and nitrogen regulation (*ntrA*, Ronson *et al.* 1987). We have also mapped the *pho-1* (Long *et al.* 1988) and *degP* (J. Glazebrook, A. Ichige, and G. C. Walker, unpublished) loci.

MATERIALS AND METHODS

Strains and media. Strains are listed in Table 1. *R. meliloti* was grown either in LB medium (Maniatis *et al.* 1982) supplemented with 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$ or in M9 medium (Maniatis *et al.* 1982) supplemented with 0.4% glucose, 1 mM $MgSO_4$, 0.25 mM $CaCl_2$, and 1 mM biotin. When appropriate, amino acids were added to supplemented M9 medium at a concentration of 20 μg/ml. *Escherichia coli* (Migula) Castellani and Chalmers MT616 was grown in LB medium containing 10 μg/ml of chloramphenicol. Antibiotics were used at the following concentrations: streptomycin (Sm), 400 μg/ml; rifampicin (Rf), 50 μg/ml; neomycin (Nm), 200 μg/ml; gentamycin (Gm), 20 μg/ml; and spectinomycin (Sp), 50 μg/ml.

Genetic techniques. Transductions were performed by using ΦM12 as described (Finan *et al.* 1984). Triparental matings using *E. coli* strain MT616, which carries plasmid

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pRK600 to provide transfer functions, were carried out as described previously (Leigh *et al.* 1985). When necessary, the antibiotic resistance genes of Tn*5* or Tn*phoA* insertions were exchanged by homologous recombination as described (de Vos *et al.* 1986). Transposon insertions co-transducible with the *pho-1* locus were isolated as previously described (Glazebrook and Walker 1991).

RESULTS AND DISCUSSION

To construct a set of Tn*5*-Mob insertion strains convenient for mapping transposon insertion mutations, we transduced six of the Tn*5*-Mob insertions constructed by Klein *et al.* (1992) into strain Rm5000, which is rifampicin resistant (Rf^r) and streptomycin sensitive (Sm^s). The Tn*5*-Mob insertions are located at several positions in the chromosome; insertions 601 and 602 are co-transducible with *leu-53*$^+$, insertions 611 and 612 are co-transducible with *trp-33*$^+$, and insertions 614 and 615 are co-transducible with *pyr-49*$^+$. The insertions in each pair are in opposite orientations, so they facilitate transfer of chromosomal markers in opposite directions (Fig. 1). Mutations to be mapped were transduced into each of these six Tn*5*-Mob-containing strains. The mutations were then mobilized from each of these six strains into Rm1021 (Sm^r), and transconjugants were obtained by selection for Sm^r and the drug resistance of the transposon marking the mutation of interest. A mutation was judged to lie between the positions of the two Tn*5*-Mob insertions from which it could be transferred at the highest frequencies.

As an example of this method, we describe the mapping of the *exoR95*::Tn*5* mutation. This mutation could not be introduced into the Tn*5*-Mob-containing strains directly, because the drug marker in Tn*5*, neomycin resistance (Nm^r), is the same as that in Tn*5*-Mob. Therefore, we used an allele in which the Tn*5* insertion in *exoR95* had been exchanged for a Tn*5-233* insertion. This *exoR95*::Tn*5-233* allele was transduced into each of the six mapping strains with selection for the gentamycin-spectinomycin resistance (Gm^rSp^r) encoded by the transposon. Six triparental matings were then performed with one of the *exoR*-containing Tn*5*-Mob strains as one parent; the *E. coli* strain MT616 provided transfer functions, and Rm1021 was the recipient. Transconjugants were obtained by selection for Gm^rSp^r and Sm^r. The frequency with which *exoR95*::Tn*5-233* was transferred from each Tn*5*-Mob strain was measured as the fraction of Sm^r colonies (representing total recipients) that were also Gm^rSp^r. We found that *exoR* was transferred more efficiently from Tn*5*-MobΩ601 (6.1×10^{-7}) and Tn5-MobΩ614 (5.8×10^{-7}) than it was from any of the other Tn*5*-Mob insertions ($<5 \times 10^{-8}$). We concluded that *exoR* lies in the region of the chromosome, between insertion 601 and insertion 614, that is transferred early from each of these origins.

Table 1. Strains used in this study

Strain	Relevant genotype	Source or reference[a]
Rhizobium meliloti		
Rm1021	*str-21*	F. Ausubel
Rm2111	*str-3*, *cys-11*	H. Meade
Rm5000	*rif-5*	T. Finan
Rm3348	*str-3*, *spc-1*, *rif-1*, *his-39*, *ilv-48*	H. Meade
Rm3351	*str-3*, *spc-1*, *rif-1*, *his-39*, *trp-33*, *aro-51*	H. Meade
Rm3357	*str-3*, *spc-1*, *rif-1*, *nov-57*, *trp-33*, *his-39*, *leu-53*	H. Meade
Rm3359	*str-3*, *spc-1*, *rif-1*, *nov-59*, *trp-33*, *his-39*, *pyr-49*	H. Meade
Rm5050	*str-21*, *met-1023*::Tn*5*	S. Long
Rm6027	*str-21*, *exoD27*::Tn*5-233*	de Vos *et al.* 1986
Rm6826	*str-3*, *spc-1*, *rif-1*, *his-39*, *trp-33*, *aro-51*, *ura-501*::Tn*5*	Klein *et al.*, in press
Rm7558	*lpsB*::Tn*5*-104-3	Clover *et al.* 1989
Rm8002	*str-21*, *pho-1*	Long *et al.* 1988
Rm8295	*str-21*, *pho-1*, *exoR95*::Tn*5-233*	Reuber *et al.* 1991
Rm8296	*str-21*, *pho-1*, *exoS96*::Tn*5-233*	Reuber *et al.* 1991
Rm8519	*str-21*, *ndvB*- TY7::Tn*5-233*	Reed and Walker 1991
Rm8608	*rif-5*, Tn*5*-MobΩ601	This work
Rm8609	*rif-5*, Tn*5*-MobΩ602	This work
Rm8610	*rif-5*, Tn*5*-MobΩ611	This work
Rm8611	*rif-5*, Tn*5*-MobΩ612	This work
Rm8612	*rif-5*, Tn*5*-MobΩ614	This work
Rm8613	*rif-5*, Tn*5*-MobΩ615	This work
Rm8614	*str-21*, *lpsB*::Tn*5*-104-3	This work
Rm8615[b]	*str-21*, *fix-382*::Tn*phoA-233*	This work
Rm8616[b]	*str-21*, *fix-383*::Tn*phoA-233*	This work
Rm8617[b]	*str-21*, *fix-379*::Tn*phoA-233*	This work
Rm8618[b]	*str-21*, *fix-385*::Tn*phoA-233*	This work
Rm8619[b]	*str-21*, *fix-388*::Tn*phoA-233*	This work
Rm8620	*str-21*, Δ*degP65*::Tn*3HoKm*	J. Glazebrook
Rm8621	*str-21*, Δ*trpE(G)621*::Sp	Barsomian *et al.* in press
A1681	*str-21*, *ntrA*::Tn*5*	F. Ausubel
Escherichia coli		
MT616	MT607 pRK600	Finan *et al.* 1986

[a] F. Ausubel, Massachusetts General Hospital; S. Long, Stanford University; H. Meade and T. Finan, Massachusetts Institute of Technology.
[b] These strains were obtained by recombination of the corresponding Tn*phoA* insertion mutants with Tn*5-233*, resulting in replacement of the sequences encoding Nm^r with sequences encoding $GmSp^r$ (de Vos *et al.* 1986). All strains are derivatives of SU47 (Vincent 1941).

We next determined the position of *exoR* relative to those of known markers in the Tn*5*-MobΩ601–Tn*5*-MobΩ614 interval. Our approach to this problem takes advantage of the fact that markers transferred early from a given origin are transferred at a much higher frequency than those transferred late. Consider two markers X and Y, which lie between transfer origins 1 and 2. Marker X lies closer to origin 1 than does marker Y. When transfer initiates at origin 1 and transconjugants are selected for X and scored for Y, many of the transconjugants will not have received marker Y in the conjugation, and the observed frequency of co-transfer of X and Y will be relatively low. If marker X is transferred from origin 2, which transfers Y before X, all transconjugants selected for X will have received Y in the conjugation, and the observed frequency with which the two markers are transferred will be relatively high.

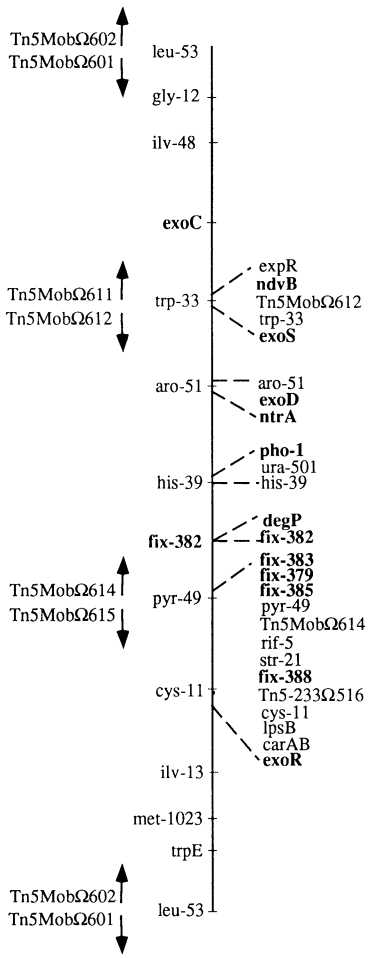

Fig. 1. Genetic map of the *Rhizobium meliloti* chromosome. The circular chromosome has been drawn in a linear fashion for convenience. The directions of transfer from Tn*5*-Mob insertions are indicated by arrows. For example, *trpE* and *met1023* are transferred early from Tn*5*-MobΩ601, whereas *gly-12* and *ilv-48* are transferred early from Tn*5*-MobΩ602. Markers that have not been linked to each other by transduction are shown to the left of the vertical line. Markers that are in co-transductional linkage groups are shown to the right. Detailed information about the transductional linkage groups is presented in Figure 2. Markers shown in boldface were either mapped *de novo* or mapped more precisely, as part of this work. The map positions of the markers not shown in boldface were determined by the following: *leu-53*, *ilv-48*, *trp-33*, *aro-51*, *ura-50l*, *his-39*, *pyr-49*, *cys-11*, *ilv-13*, *met-1023*, *trpE* (same locus as *trp-99*); and all of the Tn*5*-Mob insertions (Klein *et al.* 1992); *gly-12* (Meade and Signer 1977); *expR* (Glazebrook and Walker 1989); *rif-5*, *str-21*, and Tn*5*-233Ω516 (Williams and Signer 1987); *lpsB* and *carAB* (Clover 1988). This map is not meant to be all-inclusive. The markers shown were chosen for their utility as points of reference.

Thus, to further define the position of *exoR* within the Tn*5*-MobΩ601–Tn*5*-MobΩ614 interval, we transferred *exoR95*::Tn*5-233* from Tn*5*-MobΩ601 and from Tn*5*-MobΩ614 into strains carrying other mutations in this interval and assessed the frequency at which the wild-type alleles of the markers in the recipient strains were co-transferred with the *exoR95*::Tn*5-233* mutation. The *exoR95*::Tn*5-233* mutation was transferred from Tn*5*-MobΩ601 and from Tn*5*-MobΩ614 into Rm5050, which contains the *met-1023*::Tn*5* mutation. Transconjugants were obtained by selecting Gm^rSp^r, Sm^r colonies. These colonies were then screened for Nm^s to determine how many had lost the *met-1023*::Tn*5* mutation (i.e., received the Met^+ allele). We found that 11% of the colonies that received *exoR* from the Tn*5*-MobΩ601 strain were Nm^s, whereas only 1% of the colonies that received *exoR* from the Tn*5*-MobΩ614 strain were Nm^s. This indicated that the *exoR* locus was between *met-1023*::Tn*5* and Tn*5*-MobΩ614. We then performed a similar experiment by using as a recipient strain, Rm8614, which contains an *lpsB*::Tn*5* mutation. We found that 86% of the colonies that received *exoR* from the Tn*5*-MobΩ601 strain were Nm^s, whereas 96% of those that had received it from the Tn*5*-MobΩ614 strain were Nm^s, indicating that *exoR* was between *lpsB* and Tn*5*-MobΩ601. Taken together, these results showed that *exoR* was located between *lpsB* and *met-1023*.

The high frequency of co-transfer of *exoR* and $lpsB^+$ led us to test if *exoR* and *lpsB* were co-transducible by ΦM12. When the *lpsB*::Tn*5* allele was transduced into strain Rm8295 (*exoR95*::Tn*5-233*), 71 of the 176 Nm^r transductants obtained were Gm^sSp^s, indicating 40% co-transduction of the *exoR* and *lpsB* loci. From all the data concerning the map position of *exoR*, we concluded that it is 40% co-transducible with *lpsB* on the side toward *ilv-13* and *met-1023*.

The positions of the other loci mapped in this work (shown in bold type in Figs. 1,2) were determined in a manner similar to that used for *exoR*. We have found that when transducing mutations to be mapped into the Tn*5*-Mob strains, it is advisable to screen the transductants for loss of the Tn*5*-Mob insertions. Such losses indicate co-transduction between the mutation of interest and one of the Tn*5*-Mob insertions. We detected co-transduction of six of the markers mapped in this work with various Tn*5*-Mob insertions, which simplified the mapping of these markers considerably.

If the marker of interest is located very close to the Sm^s allele in the Tn*5*-Mob-containing donor strains, misleading results are obtained from the conjugal transfer experiments we have described. Most of the recipients that receive the marker of interest also receive the Sm^s allele from the donor and are lost, because transconjugants are selected for Sm^r. This results in artificially low frequencies of transfer. There is a very simple solution to this problem. The Sm^s allele is closely linked to Tn*5*-MobΩ614, so any markers close

to the Sm^s allele are also close to Tn*5*-MobΩ614. Therefore, if co-transduction of the marker of interest and Tn*5*-MobΩ614 is checked for during construction of the donor strains, as suggested above, markers close to the Sm^s locus are identified before conjugational mapping experiments are performed. Of course, conjugational mapping of such markers is unnecessary, because markers can be placed on the map based on the frequencies with which they are co-transduced with the Sm^r and Tn*5*-MobΩ614 mutations.

Markers that lack a selectable phenotype can be mapped indirectly by isolating a transposon insertion that is co-transducible with the marker of interest and then using the Tn*5*-Mob system to determine the position of the linked transposon. For example, to map the *pho-1* mutation, a pool of random Tn*5-233* insertions was transduced into the *pho-1* strain Rm8002, and transductants were screened for those that were Pho$^+$. Approximately 1% of the transductants were Pho$^+$. One of the Tn*5-233* insertions that was linked to the *pho-1* locus was then mapped by conjugational transfer, and eventually it was possible to perform a three-factor cross by ΦM12 transduction that ordered the *pho-1* mutation relative to the *ura-501*::Tn*5* and *his-39* mutations (Fig. 2).

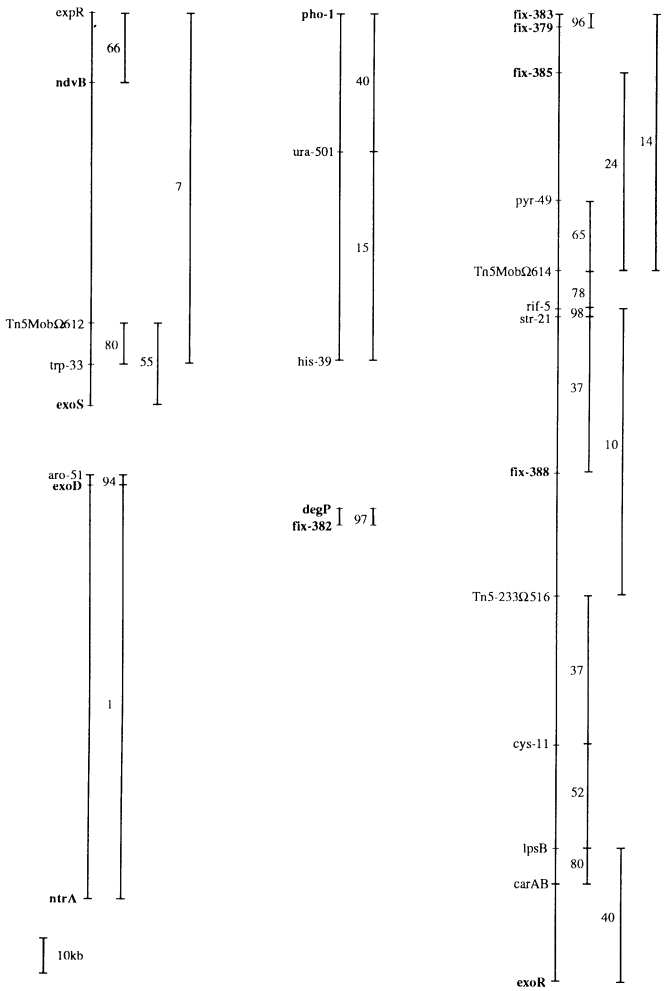

Fig. 2. Co-transductional linkage groups on the *Rhizobium meliloti* chromosome. Markers mapped in this work are shown in boldface. The numbers shown are the percentage of co-transduction by ΦM12. These co-transduction frequencies were converted to distances in kilobases by using the Wu equation (Wu 1966), assuming a length of 160 kilobases for ΦM12 (Finan *et al.* 1984). The distances between the markers were then drawn on a kilobase scale.

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