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Site Specific Transposition of Tn7 into a *Rhizobium meliloti* Megaplasmid

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Summary. Transposon Tn7 was shown to insert specifically into the megaplasmid of different *Rhizobium meliloti* strains. Tn7 transposition could not be detected in other *Rhizobium* strains such as *R. trifolii*, *R. leguminosarum*, *R. phaseoli* and *R. japonicum*. In *R. meliloti* strains, two unique sites in the megaplasmid were observed into which Tn7 can transpose at different frequencies. Only one copy of Tn7 could be detected in the megaplasmid and the insertion sites for Tn7 are outside the *nif* and *nod* region. Tn7 transposition in *R. meliloti* showed a marked preference for sites on plasmid RP4 compared to the megaplasmid sites. Attempts to cure Tn7 from the megaplasmid were unsuccessful. This site specific transposition of Tn7 in *R. meliloti* provides an additional genetic tool to further manipulate this important plasmid in symbiotic nitrogen fixation.

Introduction

Plasmids of large molecular size appear to be ubiquitous in nitrogen-fixing *Rhizobium spp.* (Nuti et al. 1977; Casse et al. 1979; Hirsch et al. 1980). Considerable variations have been detected in the physical properties of plasmids from different *Rhizobium* strains except in the case of *R. meliloti* where a megaplasmid of greater than 300 Mdal is highly conserved (Banfalvi et al. 1981; Rosenberg et al. 1981). Extensive research on the role of these plasmids has shown that they harbour genes for nodulation (*nod*) and nitrogen-fixation (*nif*) (Johnston et al. 1978; Nuti et al. 1979; Prakash et al. 1980; Ruvkun and Ausubel 1980; Prakash et al. 1981). In *R. meliloti* these functions are carried on the megaplasmid (Banfalvi et al. 1981; Rosenberg et al. 1981; Kondorosi et al. 1982) but the coding capacity and genetic functions associated with the remainder of the megaplasmid are unknown. Its high degree of conservation suggests that it may encode other vital or selectably favourable functions.

To facilitate further analysis in the absence of a readily selectable phenotype, transposons have been used to label the megaplasmid primarily in the *nif* and *nod* regions (Meade et al. 1982; Forrai et al. 1983). It is more difficult to label the megaplasmid in other regions with transposons because of the absence of selectable phenotypes.

The transposon Tn7 codes for resistance to trimethoprim, streptomycin and spectinomycin, antibiotics to which *R. meliloti* has a low intrinsic level of resistance thus facilitating a suitable selection for Tn7 in these bacteria. The transposition of Tn7 in some hosts shows a high degree of specificity for its site of insertion. In *E. coli* (Lichtenstein and Brenner 1981) and *Caulobacter* (Ely 1982) there is a single chromosomal site into which Tn7 inserts while in *Agrobacterium* (Hernalsteens et al. 1978) and *Vibrio sp.* (Thompson et al. 1981) transposition is less specific. In this paper the transposition of Tn7 is shown to be specific for the megaplasmid in *R. meliloti* thus giving this transposon the unique property of specifically labelling this key plasmid in these agronomically important root nodule bacteria.

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Materials and Methods

Bacterial Strains and Growth Media. E. coli strains used were W3110 *thy deo*C; C600 *thr leu thi lac*Y; HB101 *pro leu thi lac*Y Str^r *endo* I *rec A, hsd* R, *hsd* M (pRK2013) (all obtained from D. Helinski). *E. coli* CR34 *thy* (RP4) and W3110::Tn7 were obtained from J. Mielenz and P. Barth, respectively. *R. meliloti* strains 41, 102F34, 104B5 and E28 were described previously (Manian and O'Gara 1982). 41 Rif^r, 102F34 Rif^r and 104B5 Rif^r were isolated as spontaneous mutants resistant to 100 μg/ml of Rifampicin. *R. meliloti* E28 Cm^r was isolated as a spontaneous mutant resistant to 50 μg/ml of chloramphenicol. *R. leguminosarum* 897 Rif^r was obtained from L.K. Dunican. *R. japonicum* 110 was described previously (O'Gara and Shanmugam 1976) and 110 Gm^r was isolated as a spontaneous mutant resistant to 100 μg/ml of gentamicin. *R. phaseoli* 3644 was obtained from J. Beringer and 3644 Rif^r was a spontaneous mutant resistant to 100 μg/ml of rifampicin. *R. trifolii* CT11 Rif^r was a spontaneous mutant resistant to 100 μg/ml rifampicin and CT11 was an indigenous isolate from Irish soils. Media for the growth of *Rhizobium* strains were described previously (Manian and O'Gara 1982). *E. coli* was grown in L.B. (Miller 1972) or M9 minimal medium (Figurski et al. 1976).

Genetic Techniques. Bacterial conjugations were performed by mixing donor and recipient cells (10^9 cells of each) and filtering the suspension onto 0.45 μ filters (Millipore). Filters were incubated at 30° C on MSY medium (Manian and O'Gara 1982) for 3–12 h. Cells were resuspended in minimal medium before plating on selective media. Plasmid pRK2013 is the helper plasmid for transfer of the cloning vector pRK290 (Ditta et al. 1980) and contains the ColE1

origin of replication and the transfer genes of RK2. It has been found to act as an efficient suicide vector plasmid for introducing transposons into *Rhizobium* (Bolton and O'Gara, unpublished). Tn7 was inserted onto pRK2013 essentially as described by Barth et al. (1978) for RP4.

DNA Isolation Procedures. Plasmid DNA was detected in *Rhizobium* and *E. coli* strains following the method of Eckhardt (1978). Supercoiled plasmid DNA was prepared by the cleared lysate technique followed by ethidium bromide-casium chloride equilibirum centrifugation (Clewell and Helinski 1969). Total DNA was isolated from *Rhizobium* strains as described previously (Mielenz et al. 1979).

Restriction Enzyme Digestions. Restriction endonuclease enzymes were purchased from Boehringer (BCL) and used according to the manufacturers specifications. Analysis of restriction fragments was performed by standard agarose gel electrophoresis (1% agarose; 40 mM Tris, 20 mM acetic acid, 2 mM Na_2 EDTA, pH 8.0) and ethidium bromide staining (Maniatis et al. 1982).

Southern Blotting and Hybridization. Plasmid or restriction endonuclease digested DNA's were transferred to nitrocellulose filters (Schleicher and Schuell) according to Southern (1975). ^{32}P-labelled hybridization probes of purified pGV26 DNA (ColE::Tn7 obtained from J. Leemans) were prepared by "nick translation" (Rigby et al. 1977) using deoxycytidine 5′-(^{32}P) triphosphate 410 Ci/mmol and the Amersham nick translation kit (PB5025). Hybridization of the probes to the nitrocellulose filters and the subsequent washings of the filters were performed as described by Denhardt (1966) and Botchan et al. (1976). After washing, the filters were exposed to Kodak X-omat film.

Plant Nodulation Tests. Alfalfa seeds (*Medicago sativa*) were surfaced sterilized, planted in a perlite-sand mix and sterilized in boiler tube assemblies (Vincent 1970). *Rhizobium* cells resuspended in MSY basal salts served as inoculum. Plants were fed the nitrogen free plant medium described by Schwinghamer (1960). Nodulated plants were assayed for nitrogenase activity by the acetylene reduction assay as previously described (Manian et al. 1982) 6–8 weeks after germination.

Results

Introduction of Transposon Tn7 into Rhizobium Strains

Transposon Tn7 was introduced into several strains of *Rhizobium* using plasmid pRK2013 as the suicide vector. Under conditions where no spontaneously occurring antibiotic resistant mutants were observed, Tn7 transconjugants of *R. meliloti* 41, *R. meliloti* E28, *R. meliloti* 102F34 and *R. meliloti* 104B5 were obtained at a frequency of approximately 10^{-6} (Table 1). In the remaining *Rhizobium* strains tested, (*R. leguminosarum* 897, *R. trifolii* CC1-11, *R. phasioli* 3644 and *R. japonicum* 110) Tn7 containing transconjugants were not detected indicating that Tn7 transfer does not occur in these strains or occurs at too low a frequency to be detected.

The vector plasmid used, pRK2013, confers resistance to kanamycin and in all the *R. meliloti* transconjugants examined (6,000) no evidence was obtained for plasmid survival or establishment in *Rhizobium*, thus indicating that Tn7 had integrated into the continuity of the genome. Insertion of Tn7 gives rise to the possibility of mutation generation in surviving clones. Tn7 transconjugants of *R. meliloti* 41 (4,000 approx) were screened for mutants. Selection was carried out for amino acid auxotrophs, vitamins requiring mutants and inability to utilise several sugars including manitol, glucose, fructose, sucrose, rafinose, arabinose, xylose, gluconate and succinate as sole source of carbon. No mutants were obtained suggesting that the transposition of Tn7 showed some degree of specificity in the genome of *R. meliloti.*

Table 1. Introduction of Tn7 into *Rhizobium spp*

Recipient	Selection	Frequency of Tn7 transfer
R. meliloti 41 Rif	Sp/Tp	3×10^{-6}
R. meliloti 102F34 Rif	Sp/Tp	2.5×10^{-6}
R. meliloti 104B5 Rif	Sp/Tp	1.5×10^{-5}
R. meliloti E28 Cm	Sp/Tp	5×10^{-6}
R. leguminosarum 897 Rif	Sp/Tp	$<10^{-8}$
R. trifolii CT11 Rif	Sp/Tp	$<10^{-8}$
R. phaseoli RCR 3644 Rif	Sp/Tp	$<10^{-8}$

Plasmid transfer mediated by conjugation and selection of Tn7 transconjugants was carried out as outlined in Materials and Methods. *E. coli* W3110 pRK2013::Tn7 was used as donor. Antibiotics Tp, Sp, and Str were used at 100 μg/ml each for *R. meliloti* strains 41 Rif, 104B5 Rif, E28Cm and *R. japonicum* 110 Gm. For Rm 102F34 Tp concentration was 300 μg/ml. For *R. leguminosarum* 897, *R. trifolii* CT11 and *R. phaseoli* 3644 Rif, Tp, Sp, and Str were used at 50 μg/ml each

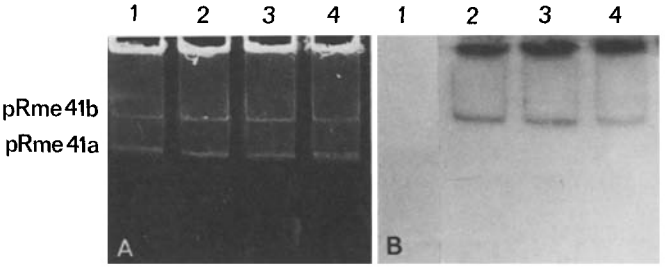

Fig. 1A, B. Hybridization of ^{32}P-labelled Tn7 to megaplasmid (pRme 41b) in *R. meliloti* 41::Tn7. **A** Plasmid pRme 41a and pRme 41b were separated on 0.7% agarose gels as described in Methods. Lane 1 *R. meliloti* 41 (WT). Lanes 2–4, *R. meliloti* 41::Tn7 (separate isolates). **B** Autoradiogram of hybridization of Tn7 probe to the tracks described in (A) following procedure outlined in the Methods

Transposition of Tn7 to the Megaplasmid of R. meliloti

The observation that Tn7 encoded resistances could only be detected in *R. meliloti* (Table 1), coupled with the failure to detect Tn7 induced mutants of *R. meliloti* 41 suggested that transposition was specific for a site(s) in *R. meliloti* unique to these strains. To test this possibility the plasmids of independent isolates of *R. meliloti* 41 harbouring Tn7 were separated and probed with ^{32}P-labelled Tn7 DNA (Fig. 1). In all cases examined the megaplasmid (pRme 41b)

of *R. meliloti* 41::Tn7 showed hybridisation to labelled Tn7. As the megaplasmid is highly conserved in all *R. meliloti* strains and since only these strains acted as hosts for Tn7, the megaplasmids of the other *R. meliloti* strains (*R. meliloti* E28, *R. meliloti* 102F34 and *R. meliloti* 104B5) were examined for the presence of Tn7. The results obtained showed that in all cases, regardless of strain, Tn7 had inserted into the megaplasmid of these strains. These results demonstrate that invariably Tn7 transposes into the megaplasmids.

Specificity of Tn7 Insertion into the Megaplasmid

Despite the fact that Tn7 has been shown to insert into the megaplasmid, there may be several sites on this plasmid into which Tn7 can transpose or there may also be more than a single copy of Tn7 per cell. To examine these possibilities total DNA from independent *R. meliloti* 41::Tn7 strains was isolated, restricted with EcoR1 and probed with ^{32}P-labelled Tn7 DNA. EcoR1 has a single asymmetric site on Tn7 so that two bands containing Tn7 DNA are produced on digestion. The results obtained (Fig. 2) show that two distinct patterns are produced. Both patterns show that only a single copy of Tn7 is present. In over 90% of cases the pattern obtained was identical where two DNA fragments containing Tn7 DNA were obtained and separated on gels. In the remaining 10% of Tn7 transconjugants, the Tn7 containing DNA fragments produced were so similar in size that they co-migrated under the electrophoresis conditions used (lane 5, Fig. 2). The presence of these distinct patterns suggests that either (i) there are two sites in *R. meliloti* 41 into which Tn7 inserts or (ii) that Tn7 inserts in opposite orientations at a unique site and because of the single asymmetric EcoR1 site two distinct restriction patterns are obtained. If this latter possibility was correct then the combined size of the fragments carrying Tn7 DNA should be similar regardless of orientation. Analysis of the patterns in Fig. 2 shows that this cannot be the case since the larger band in lanes 2–4; 5–7 co-migrates with the doublet of lane 5. The main conclusion from these experiments is that there are two sites on the megaplasmid into which Tn7 can transpose.

A similar analysis was carried out on the Tn7 trasconjugants of the other three *R. meliloti* strains studied. The results obtained for these strains were identical to those obtained for *R. meliloti* 41. These results show that Tn7 transposition in *R. meliloti* is specific for one of two sites in the megaplasmid regardless of the strain in which Tn7 is transposing.

Retransposition of Tn7 from the Megaplasmids

To show that the transposition properties of Tn7 located in the megaplasmid of *R. meliloti* 41 were intact, its ability to retranspose from this site to the plasmid RP4 was examined (Table 2). RP4 was introduced into *R. meliloti* pRme 41b::Tn7 by conjugation (frequency of 5×10^{-3}). Transposition of Tn7 to RP4 was detected by (i) selecting for transfer of RP4 to *E. coli* C600 Nal^r and screening for co-transfer of Tn7 encoded resistances and (ii) by direct selection of Tn7 markers. Transfer of RP4 was obtained at a frequency of 7×10^{-3} while co-transfer of Tn7 occurred at a frequency of 2×10^{-7}. These results indicate that while transposition of Tn7 from the megaplasmid is possible it occurs at a low frequency. In all cases examined the size of RP4::Tn7 plasmids in *E. coli* C600 Nal^r was consistant with the precise transposition of Tn7 from the megaplasmid to RP4. In no case was the megaplasmid mobilized from *R. meliloti* pRme 41b::Tn7 to *E. coli* C600 Nal^r.

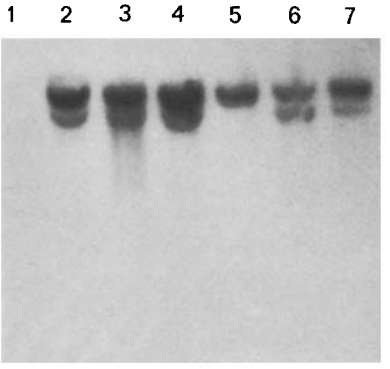

Fig. 2. Autoradiogram of hybridization of Tn7 DNA to EcoR1 digested total DNA from independent isolates of *R. meliloti* pRme 41b::Tn7 (tracks 2–7; *R. meliloti* 41 (WT) track 1. Hybridization was carried out as described in Methods

Table 2. Retransposition of Tn7 in *Rhizobium meliloti*

Donor	Recipient	Selection	Frequency of transfer
E. coli CSH 29 (RP4)	*R. meliloti* (pRme 41b::Tn7)	Tc	5×10^{-3}
R. meliloti pRme 41b::Tn7 (RP4)	*E. coli* C600	Nm	7×10^{-3}
		Tp/Sp	2×10^{-7}

Plasmid transfer and detection of Tn7 transposition was carried out as described in Mateterials and Methods. Antibiotics concentrations were Tc (20 μg/ml), Nm (100 μg/ml), Tp (50 μg/ml), Sp (50 μg/ml)

Affinity of Tn7 for the Megaplasmid in the Presence of Alternative Sites

Results presented demonstrate that Tn7 can transpose to two sites on the megaplasmid of *R. meliloti* but shows a 90% preference for one of these sites (Fig. 2). The possibility remains however, that Tn7 can transpose to other sites on the *Rhizobium* genome but because of its high affinity for one of these sites its transpositon to other site(s) was not detected. To examine the preference of Tn7 for this site, alternative sites into which Tn7 is known to transpose were introduced into the *R. meliloti* background in the frequency of Tn7 transposition to the megaplasmid was examined. These alternative sites were introduced by transferring plasmid RP4 into *R. meliloti* 41 and subsequently introducing Tn7. Transfer of Tn7 to *R. meliloti* 41 (RP4) occurred at a frequency of 2.5×10^{-5}, approximately 10 fold higher than when *R. meliloti* 41 served as the recipient. Transposition of Tn7 onto RP4 was detected by screening for co-transfer of Tn7 and RP4 markers to *E. coli* C600. Since transfer of Tn7 to *E. coli* will only occur when it is carried by the RP4 plasmid (see above) and retransposition from the megaplasmid to RP4 occurs at a low frequency (Ta-

ble 2), detection of Tn7 transposition to RP4 can be achieved by screening for transfer of Tn7. From the 1,000 clones examined, Tn7 was found to preferentially transpose to RP4 with 100% efficiency.

These results could be consistant with their being two copies of Tn7 per cell with one of these on RP4. To test for the presence of a second copy of Tn7 on the megaplasmid the plasmids of these strains were probed with ^{32}P-labelled Tn7 DNA. Hybridisation was obtained only to the RP4::Tn7 plasmid and not to the megaplasmid showing the presence of a single copy of Tn7. This finding was confirmed by probing EcoRI digests of total DNA from these isolates with ^{32}P-labelled Tn7. The pattern obtained was differnt from that obtained previously when *R. meliloti* pRme 41b::Tn7 was treated similarly (Fig. 2). These results show that there is a single copy of Tn7 per cell and this is always located on RP4 rather than on the megaplasmid. Thus Tn7 shows a preference for sites on RP4 over those on the megaplasmid.

During the course of examining *R. meliloti* 41 (RP4/Tn7) strains different plasmid classes were detected. In the majority of cases the Tn7 transposed from pRK2013::Tn7 to RP4 but in a minority of transconjugants, interactions between the RP4 and the vector plasmid occurred forming either a single composite plasmid which carried Tn7 or two independent replicons. In all cases no evidence was obtained for a copy of Tn7 on the megaplasmid. A fourth class of transconjugants was detected at low frequency (approx 1%). These showed the same plasmid profile as the major class (RP4::Tn7) but showed only between 55% and 75% cotransfer of Tn7 with RP4 markers. Hybridization analysis showed that these plasmids did in fact carry a copy of Tn7 and the low frequency of cotransfers may reflect the site on the RP4 into which Tn7 had transposed.

Plant Tests on R. meliloti Containing Tn7

Nif and *Nod* genes in *R. meliloti* are located on the megaplasmid. The finding that Tn7 transposes uniquely into this plasmid raises the possibility of specifically labelling these genes. The Tn7 containing *R. meliloti* clones were used to inoculate alfalfa plants and to examine their nodulation and nitrogen fixation properties. In all cases tested these strains were able to nodulate plants and promote nitrogen fixation as tested by the acetylene reduction test. These results indicate therefore, that the site of Tn7 integration in the megaplasmid is not in the *Nif* or *Nod* region, but occurs in some other uncharacterised region of the megaplasmid.

Discussion

Transposon Tn7 can insert specifically into particular sites in the genomes of different bacteria. In the case of *Rhizobium spp.* results presented in this communication demonstrate that transposition of Tn7 can only be detected in *R. meliloti* where Tn7 transposes specifically into the megaplasmid of these strains. The transposition frequency observed in *R. meliloti* is low compared to other hosts such as *E. coli* (Lichtenstein and Brenner 1981). The inability to detect Tn7 transposition in *Rhizobium* strains other than *R. meliloti* may reflect either (1) the presence of unique sequences located on the megaplasmid of *R. meliloti* and not present in other rhizobial strains or (2) inefficient transposition rather than Tn7 not having appropriate target site(s) for transposition. The affinity of Tn7 for the identified target sites on the megaplasmid is low compared to the sites on plasmid RP4. When RP4 is present in an *R. meliloti* background, the transposition frequency of Tn7 increases 10 fold. If a similar situation occurs in other *Rhizobia* where less efficient sites are present, then transposition frequencies may be too low to be readily detected. Furthermore, the inability to detect transposition of Tn7 in these *Rhizobium* strains is unlikely to be the result of failure of the vector plasmid pRK2013 to transfer into these hosts. This plasmid carries the transfer genes of RK2 an IncP group plasmid which transfers at high frequency to *Rhizobium spp.* (Ditta et al. 1980).

The observation that Tn7 transposes preferentially to one of the two identified sites in the megaplasmid and also shows a marked affinity for sites on RP4 relative to megaplasmid sites, suggests that Tn7 can choose from a gradient of preferential integration sites in *R. meliloti.* Similar observations on preferential integration into specific sites in the genome of *Vibrio* (Thompson et al. 1981) has also been reported. In some hosts however, Tn7 transposition is much less specific (Merrick et al. 1980; Hermalsterns et al. 1978) and indications are that the host strain may play a role in determining transposition properties of Tn7. Evidence for this in the case of *R. meliloti* comes from the observaton that Tn7 can transpose apparently less specifically into segments of the megaplasmid incorporated into plasmid RP4 in an *E. coli* background (Denarié 1982) compared to the behaviour of Tn7 in *R. meliloti* reported in this study. The role of the host as reflected in the different transposition properties of Tn7 may involve variations in the transcription efficiency of the genes encoded on the transposon or alternatively Tn7 may require a particular factor(s) supplied by the host cell.

Segments of the megaplasmid (*Nod* and *Nif* regions) can be lost by temperature curing (Banfalvi et al. 1981). Attempts to cure Tn7 from one of the *R. meliloti* 41::Tn7 clones failed under conditions where RP4 was cured with approximately 80% efficiency. Failure to cure Tn7 is unlikely to be the result of retransposition to other sites in view of the low transposition frequencies observed in these strains unless elevated temperature (39° C) affects its transposition properties. Alternatively Tn7 may be inserted near a region which if lost through imprecise excision of Tn7 or elimination of the plasmid renders the cell inviable through loss of essential function(s). Further work is in progress to determine the nature of the sites on the megaplasmid into which Tn7 inserts.

The transposition properties of Tn7 in *R. meliloti* are such that it specifically labels a region of the megaplasmid which up to now has been uncharacterized. The site of Tn7 insertion in the megaplasmid is outside the Nif and Nod regions as shown by the ability of *R. meliloti* 41::Tn7 strains to nodulate alfalfa plants and promote nitrogen-fixation. This property of Tn7 provides a positive selection which can be used to further manipulate this region of the *Rhizobium* genome. It can be used as a tool in classical genetic analysis and also as a selection for the identification and physical analysis of the DNA on this vital plasmid for symbiotic nitrogen fixation.

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