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Rhizobium meliloti Genes for Exopolysaccharide Synthesis and Nodule Infection Located on Megaplasmid 2 Are Actively Transcribed during Symbiosis

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A 7.8-kb DNA fragment of the second megaplasmid of *Rhizobium meliloti* 2011 known to carry genes for the synthesis of acidic exopolysaccharide (EPS) and for nodule infection (Inf) was mutagenized in *Escherichia coli* by transposon Tn*5*-B20. Transfer of the mutagenized fragment to *R. meliloti* resulted in the construction of merodiploid and homogenotized strains. Of eight homogenotized strains, five turned out to be of the Inf⁻ EPS⁻ type. Four of them were mutated in a region characterized by the *R. meliloti* mutant 0540 (Müller, Hynes, Kapp, Niehaus, and Pühler 1988, Mol. Gen. Genet. 211:17). The fifth Inf⁻ EPS⁻ mutant (Rm154), mutated in a different region, could be stained with Congo red and aniline blue, indicating an unusual surface polysaccharide. Two other mutants of this second region exhibited no defects in symbiosis but differed from the wild type in the level of EPS production. The promoterless *lacZ* gene of transposon Tn*5*-B20 was used to monitor the transcriptional activity at the insertion sites of merodiploid and homogenotized *R. meliloti* strains in the free-living as well as in the bacteroid state. Three regions showed a high level of transcription in the free-living state. The transcription of these regions was not switched off during symbiosis.

Additional keywords: fragment-specific mutagenesis, *lacZ* fusion, polysaccharides, Tn*5*-*lacZ*.

Soil bacteria of the genus *Rhizobium* induce nitrogen-fixing nodules on the roots of leguminous plants. During establishment of symbiosis, signal molecules are exchanged between the plant and the microsymbiont. These molecules play an important role for the specificity of the interaction (Halverson and Stacey 1986; Djordjevic *et al.* 1987). The acidic exopolysaccharide (EPS) of *R. meliloti* is thought to be essential for nodule invasion. Mutants of *R. meliloti* unable to produce EPS showed defects in nodule invasion. They were no longer able to form infection threads (Inf⁻) (Finan *et al.* 1985; Leigh *et al.* 1985; Hynes *et al.* 1986; Müller *et al.* 1988). Genes affected in these mutants have been demonstrated to be located on the second megaplasmid of *R. meliloti* (Finan *et al.* 1986; Hynes *et al.* 1986; Leigh *et al.* 1987).

We recently reported that the Tn*5* insertion sites of two Inf⁻ EPS⁻ *R. meliloti* mutants (0540 and 2505) are located on a 7.8-kb fragment of the second megaplasmid (Müller *et al.* 1988). In this paper we now report a more detailed analysis of this 7.8-kb fragment. The genetic analysis was carried out by fragment-specific mutagenesis in *E. coli* by using transposon Tn*5*-B20. This transposon carries a promoterless *lacZ* gene that allows the transcriptional activity at the transposon insertion site to be monitored.

MATERIAL AND METHODS

Bacteria and plasmids. Strains and plasmids used in this work are listed in Table 1.

Media and growth conditions. Either tryptone-yeast (TY) medium (Aguilar *et al.* 1985), Vincent medium (Vincent 1970), or Luria-Bertani (LB) medium (Maniatis *et al.* 1982) was used for bacterial growth. LBMM medium was freshly prepared by adding 1 g/L of maltose and 2 g/L of $MgCl_2$ to LB medium. Rhizobium minimal medium (RMM; pH 7.4) contained, per liter, 10 g of mannitol, 2.05 g of K_2HPO_4, 1.45 g of KH_2PO_4, 0.5 g of NH_4NO_3, 0.15 g NaCl, 0.62 g of $MgSO_4 \cdot 7H_2O$ (autoclaved separately), 0.01 g of $CaCl_2$ (autoclaved separately), 6.7 mg of iron citrate (autoclaved separately); trace elements (autoclaved separately): 340 μg of $MnSO_4$, 250 μg of H_3BO_3, 290 μg of $ZnSO_4 \cdot 7H_2O$, 100 μg of $CuSO_4 \cdot 5H_2O$, 60 μg of $CoSO_4 \cdot H_2O$, 50 μg of $Na_2MoO_4 \cdot 2H_2O$, and 1 mg of vitamins (biotin, thiamine, and Ca-pantothenate, separately) per liter were added. M98 medium (pH 7.9) was composed of M9 medium (Miller 1972), supplemented per liter with 10 g of mannitol, 10 g of glucose, and 1 ml of a vitamin solution (1 mg/L of biotin, thiamine, and Ca-pantothenate). Growth conditions have been described previously (Simon 1984). For solid media 10 g of agar per liter was added. Antibiotics were added at the following final concentrations: neomycin (Nm), 100 μg/ml; kanamycin (Km), 50 μg/ml; streptomycin (Sm), 600 μg/ml; and tetracycline (Tc), 7 μg/ml.

DNA isolation and transformation of *E. coli*. Total DNA of *R. meliloti* was isolated according to the method published by Meade *et al.* (1982). Plasmid DNA was isolated as described by Priefer (1984). Transformation of *E. coli* cells with plasmid DNA was perfomed as published by Morrison (1977).

Fragment-specific mutagenesis of plasmid pRmPM157.8 in *E. coli* with Tn*5*-B20. Phage λ573 carrying the transposon Tn*5*-B20 (Fig. 1) (Simon, Klipp, Quandt, and Pühler, unpublished) was used to mutagenize the *E. coli* strain S17-1 harboring plasmid pRmPM157.8. *E. coli* S17-1 (pRmPM157.8) was grown in LBMM medium to mid log phase and infected with λ573::Tn*5*-B20 at a multiplicity of infection ranging from 0.1 to 2.0. Following infection cells were plated on LBMM agar containing Km and Tc. To select for Tn*5*-B20 mutagenized pRmPM157.8 plasmids,

single colonies were replica plated on a lawn of an *E. coli* CSH52 recipient strain. Tn*5*-B20 carrying pRmPM157.8 plasmids transferred by conjugation to the *E. coli* recipient strain could be recognized by selection for Km and Tc resistance. Plasmid DNA was isolated from the transconjugants and retransformed into *E. coli* S17-1. The location of the Tn*5*-B20 insertions in pRmPM157.8 was determined by using restriction sites introduced by Tn*5*-B20. Because *Eco*RI, *Xho*I, and *Hin*dIII cleave Tn*5*-B20 DNA asymmetrically, digestion with these enzymes allowed the orientation of the inserted Tn*5*-B20 transposon to be determined.

Transfer of plasmids between *E. coli* and *R. meliloti*. *E. coli* S17-1 was used to transfer the hybrid plasmids pRmPM157.8::Tn*5*-B20 to *R. meliloti*. The transfer was carried out by filter matings (Simon 1984). Replacement of the *R. meliloti* wild-type fragment by the mutated fragment was performed as previously described (Aguilar *et al.* 1985), omitting the enrichment procedure with ampicillin.

Nodulation- and nitrogen-fixing assays for *R. meliloti* strains. Alfalfa seeds (*Medicago sativa* 'Du Puits') were surface sterilized and germinated as described by Müller *et al.* (1988). Inoculation of the seedlings was carried out with late log phase bacteria. The plantlets were grown on nitrogen-free medium, as described by Rolfe *et al.* (1980) and inspected after 3–5 wk. Nitrogenase activity of the nodulated plants was measured by the acetylene reduction assay (Hardy *et al.* 1968). The occurrence of infection threads was tested by the phenol-clearing method, as previously described by Niehaus and Pühler (1988).

Staining procedures of *R. meliloti* colonies. Acidic EPS production by *R. meliloti* strains was tested by plating single cells on M98 agar containing Cellufluor white, as previously described by Hynes *et al.* (1986). Production of polysaccharides different from acidic EPS was tested on Congo red (Serva) and anilin blue (Sigma Chemical Co.) containing agar consisting of Vincent or RMM medium supplemented with 0.02% Congo red or with 0.005% anilin blue.

Quantitative analysis of EPS production. Quantitative analysis of acidic EPS production was performed as described by Müller *et al.* (1988). Cells were grown for 10 days in 10 ml of Vincent medium and pelleted by centrifugation (10,000 rpm, 30 min). For precipitation of acidic EPS, the supernatant was mixed with cetylpyridinium chloride. The amount of EPS was determined by using the sulfuric acid-anthrone method (Nowotny 1969).

Electron microscopy to visualize exostructures of free-living *R. meliloti* cells. Cells of *R. meliloti* strains to be tested were grown for 1 wk in Vincent medium and stained with Ruthenium red and uranylacetate, as described by Mutaftschiev *et al.* (1982). Exostructures were viewed by using a transmission electron microscope (Hitachi HS500) operated at 75 kV.

β-galactosidase assay of free-living *R. meliloti* strains carrying Tn*5*-B20. The assay was carried out as described by Aguilar *et al.* (1985). Cell density was determined by measuring the absorbance of the cell suspension at 600 nm. The reaction was started by adding 0.4 ml of ONPG (4 mg/ml of Z-buffer) to the cell suspension. The reaction was stopped after 5–30 min by adding 1 ml of 1 M Na_2CO_3. Before measuring the optical absorbance of the reaction mixture at 420 nm, the cells were spun down. Relative β-galactosidase units were calculated per cell number according to Miller (1972).

β-galactosidase assay of *R. meliloti* bacteroids carrying Tn*5*-B20. Bacteroids were isolated from alfalfa nodules as described by Aguilar *et al.* (1985). Nodules were picked 3 wk after inoculation and their wet weight was determined. The nodules were surface sterilized with 70% ethanol, thoroughly washed with sterile distilled water, and crushed in a 0.5 M mannitol/30 mM Tris-HCl (pH 7.5) solution by using a mortar and pestle. The homogenate was transferred into an Eppendorf tube. Plant debris was pelleted by centrifugation in a Biofuge (Heraeus) for 5 min at 1,700 rpm. The supernatant was centrifuged for 3 min at 5,000 rpm to pellet the bacteroids. The pellet was resuspended in 0.4 ml of the mannitol-Tris solution. A volume of 0.2 ml of the bacteroid suspension was used for the β-galactosidase assay, which was carried out as described for free-living cells. The relative β-galactosidase activity of bacteroids was calculated per milligram of nodule wet weight.

Examination of merodiploid and homogenotized *R. meliloti* strains by hybridization experiments. Merodiploid and homogenotized *R. meliloti* strains were examined by Southern blot analysis. Total DNA of the *R. meliloti* strains was digested with *Eco*RI, and the fragments were separated

Table 1. Bacterial strains, plasmids, phage, and transposon

Designation	Relevant characteristics[a]	Origin
Escherichia coli		
CSH52	λ⁻, *str*A, *rec*A	Miller 1972
S17-1	F⁻, λ⁻, *rec*A, *hsd*R⁻ carrying a modified RP4 (Ap^s, Tc^s, Km^s) integrated in the chromosome	Simon *et al.* 1983
Rhizobium meliloti		
2011 (wild type)	Nod^+, Fix^+, EPS^+, Cfw^+; Sm^r	J. Dénarié
Plasmids, phage, and transposon		
PSUP102	pACYC184-*mob*; Tc^r, Cm^r	Simon *et al.* 1986
pRmPM157.8	pSUP102 carrying a 7.8-kb *Eco*RI fragment; Tc^r, Cm^s	Müller *et al.* 1988
λ573	*b*221 (*att*⁻, *int*⁻) red⁻, *O*am, *P*am, cI857^{ts}	N. Kleckner
Tn*5*-B20	Tn*5* carrying a promoterless *lac*Z gene cloned into IS*50*L (Fig. 1)	Simon *et al.* (unpublished)

[a] Nod = nodule induction; Fix = nitrogen fixation; EPS = exopolysaccharide production; Cfw = UV fluorescence on Cellufluor white agar; Ap = ampicillin; Tc = tetracycline; Km = kanamycin; Sm = streptomycin; Cm = chloramphenicol.

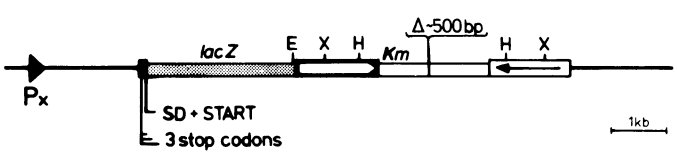


Fig. 1. Genetic map of the transposon Tn*5*-B20 carrying a promoterless *lac*Z gene. The map shows the structure of Tn*5*-B20 with the following symbols: ⟵, IS*50*R; ▭, IS*50*L; □, central region of Tn*5*-B20 with a deletion of about 500 bp to remove the *Bam*HI site; ▨, promoterless *lac*Z gene; E, *Eco*RI; H, *Hin*dIII; X, *Xho*I; P_x, the promoter to be examined by Tn*5*-B20; Km, kanamycin-resistance gene. The stop codons in all three possible reading frames in front of the Shine-Dalgarno sequence (SD) are indicated. A detailed map and a description of the construction of Tn*5*-B20 will appear elsewhere (Simon, Klipp, Quandt, Pühler, unpublished).

by agarose gel electrophoresis and transferred to nitrocellulose filters (Schleicher and Schüll) as described by Maniatis *et al.* (1982). A biotin hybridization kit (Bethesda Research Laboratories) was used according to the recommendations of the manufacturer. The labeling of the hybridization probes (Tn*5* and plasmid pRmPM157.8 DNA) was performed by nick translation with biotinylated dATP. Hybridization was carried out overnight at 42° C in 45% formamide.

RESULTS

Construction of merodiploid and homogenotized *R. meliloti* strains carrying Tn*5*-B20 mutations in a 7.8-kb DNA fragment of the second megaplasmid. A 7.8-kb *Eco*RI fragment of the *R. meliloti* megaplasmid 2 known to carry genes for EPS biosynthesis and infection of alfalfa nodules was mutagenized in *E. coli* by using transposon Tn*5*-B20 (Fig. 1). For this reason, the *E. coli* strain S17-1 (pRmPM157.8) was infected with the λ phage 573 carrying Tn*5*-B20. Tn*5*-B20 carrying pRmPM157.8 plasmids were obtained by conjugational transfer to the *E. coli* recipient strain CSH52. DNA of Tn*5*-B20 carrying pRmPM157.8 plasmids was isolated, and the Tn*5*-B20 insertion site as well as the orientation of the insertion were determined by restriction analysis. Altogether, 30 different pRmPM157.8::Tn*5*-B20 plasmids were analyzed. Fourteen of them carried the Tn*5*-B20 transposon in the cloned 7.8-kb fragment. Figure 2 shows the location and orientation of each individual Tn*5*-B20 insertion. The 14 hybrid plasmids were designated pRmMK101, pRmMK111, pRmMK116, etc. The Tn*5*-B20 insertions obtained were subdivided into four regions (Fig. 2). Region I consists of insertions in the vicinity of the previously described 2505 mutation (Müller *et al.* 1988). Region II is newly described. Insertions close to the 0540 mutation (Müller *et al.* 1988) form region III, whereas insertions close to the right end of the 7.8-kb fragment are grouped in region IV.

The hybrid pRmMK plasmids were used to introduce the Tn*5*-B20 insertions in the *R. meliloti* genome. This was accomplished by reintroduction of the individual pRmMK plasmids into the mobilizing strain *E. coli* S17-1 and by a subsequent mating with *R. meliloti* 2011. Because transferred pRmMK plasmids cannot replicate in *R. meliloti*, Tc-resistant transconjugants carry the hybrid pRmMK plasmids integrated into the genome. This integration occurs via a single crossing-over between the two copies, the 7.8-kb fragment present on pRmMK plasmids and on megaplasmid 2 of *R. meliloti* (Fig. 3A). In a further step, the merodiploid strains were used for the isolation of homogenotized *R. meliloti* strains carrying only the Tn*5*-B20 mutated 7.8-kb fragment. These homogenotized strains arise by a second crossing-over, as indicated in Figure 3B. By this procedure, several Tn*5*-B20 mutations could be introduced into the *R. meliloti* megaplasmid 2. The resulting homogenotized *R. meliloti* strains (e.g., Rm110) are listed in Figure 3B. The frequency of the second crossing-over was about 10^{-2}. For six out of 14 Tn*5*-B20 insertions, homogenotization was not achieved. Three Tn*5*-B20 insertions are located at both ends of the 7.8-kb fragment (Fig. 2). For the insertions 120, 101, and 143, several thousand colonies were screened but no homogenotized strains were found.

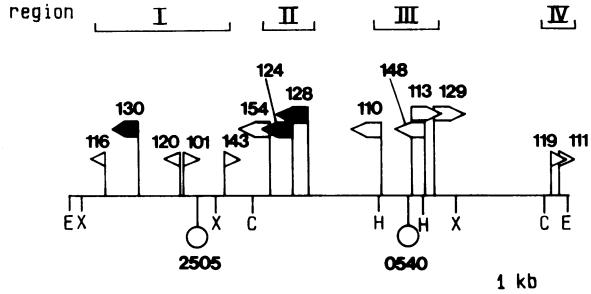


Fig. 2. Location and orientation of Tn*5*-B20 insertions in the 7.8-kb *Eco*RI fragment of the *R. meliloti* megaplasmid 2. The restriction map for *Eco*RI (E), *Xho*I (X), *Cla*I (C), and *Hin*dIII (H) of the 7.8-kb fragment of the *R. meliloti* megaplasmid 2, according to Müller *et al.* (1988), is presented. The previously mapped mutations 0540 and 2505 (○) are indicated (Müller *et al.* 1988). Tn*5*-B20 insertion sites are shown as big and small flags. The flags indicate the transcription direction of the promoterless *lac*Z gene in the Tn*5*-B20 transposon. For six Tn*5*-B20 insertions, no homogenotization was observed (small flags). For eight Tn*5*-B20 insertions, homogenotization was successful (big flags). Open big flags indicate an Inf^- EPS^- phenotype of the homogenotized *R. meliloti* strain; filled big flags indicate an Inf^+ EPS^+ phenotype of the homogenotized *R. meliloti* strain. The Tn*5*-B20 insertions were grouped in four regions (I–IV) as indicated.

All merodiploid and homogenotized strains were checked by Southern hybridization. Restricted total DNA of these strains was hybridized to pRmPM157.8 and Tn*5* DNA. The hybridization pattern confirmed the postulated single crossing-over for merodiploid or the postulated double crossing-over for homogenotized *R. meliloti* strains (data not shown). By this method, several *R. meliloti* strains were also detected that had received the Tn*5*-B20 transposon by transposition and not by homogenotization (data not shown). These strains, of course, were of no use for further investigations. Additionally, all homogenotized mutant strains described so far could be complemented by the wild-type 7.8-kb fragment on plasmid pRmPM157.8, showing that the mutations are caused solely by the Tn*5*-B20 insertions.

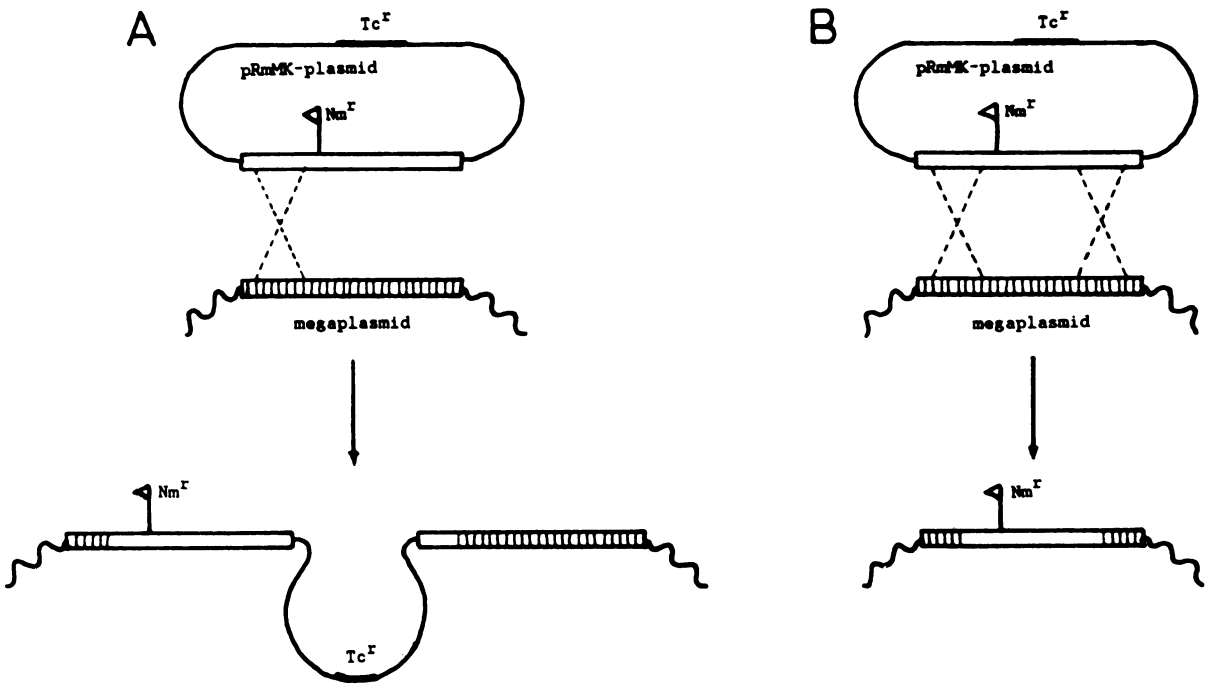
EPS production and nodule infection by the merodiploid and homogenotized *R. meliloti* strains. The homogenotized *R. meliloti* strains listed in Figure 3B were all tested for their ability to produce acidic EPS and to infect alfalfa nodules. EPS production was assayed on Cellufluor white plates and by isolation of EPS by cetylpyridinium chloride precipitation. Nodule infection was examined by the occurrence of empty or wild-type alfalfa nodules. The results are summarized in Table 2.

The homogenotized strain Rm130 of region I exhibited the wild-type phenotype with normal production of acidic EPS (EPS^+) and induction of nitrogen-fixing alfalfa nodules (Nod^+, Fix^+), indicating that the inserted Tn*5*-B20 transposon does not interfere with EPS production and nodule infection.

The four homogenotized strains Rm110, Rm148, Rm129, and Rm113 of region III carry the Tn*5*-B20 mutations in the vicinity of the Tn*5* insertion site of the previously described mutant 0540 (Fig. 2) (Müller *et al.* 1988). These four mutants showed similar phenotypes. They did not produce EPS and showed no fluorescence when stained with Cellufluor white. Furthermore, these mutants were unable to infect nodules (Nod^+, Inf^-, Fix^-). Evidently, region III, spanning about 1 kb, plays an essential role in EPS production and nodule infection.

The Tn*5*-B20 insertion of mutant Rm154 located in region II also blocks acidic EPS production and nodule infection. The surface polysaccharides produced by this mutant, however, represent a novel phenotype, which will be described in a separate section.

Although the Tn*5*-B20 insertions of the homogenotized strains Rm124 and Rm128 (region II) map in the vicinity of mutation 154, their phenotypes were completely different. Both strains were able to induce nitrogen-fixing nodules. Strain Rm124, however, was more effective in nodulation than the wild type. After 3 wk of inoculation, strain Rm124 induced an average of four N_2-fixing nodules per plant (the wild type induced only two to three). Nodules induced by strain Rm124 reached a medium length of 35 mm after 3 wk (wild-type nodules only 30 mm). Also, an increased number of leaves was observed with plants inoculated with Rm124 (five leaves after 3 wk, compared with four leaves for plants inoculated with the wild-type strain). The values reported are mean values calculated from at least 160 plants. Both strains, Rm124 and Rm128, differed in EPS production. Rm124 overproduced EPS (~ 300%), whereas Rm128 produced only 30% of the EPS normally synthesized by the wild type. These results indicate that nodule infection is not critically dependent on a certain amount of EPS.



merodiplod strain	homogenotized strain
Rm2011-pRmMK116	none
Rm2011-pRmMK130	Rm130
Rm2011-pRmMK120	none
Rm2011-pRmMK101	none
Rm2011-pRmMK143	none
Rm2011-pRmMK154	Rm154
Rm2011-pRmMK124	Rm124
Rm2011-pRmMK128	Rm128
Rm2011-pRmMK110	Rm110
Rm2011-pRmMK113	Rm113
Rm2011-pRmMK148	Rm148
Rm2011-pRmMK129	Rm129
Rm2011-pRmMK111	none
Rm2011-pRmMK119	none

Fig. 3. Construction of *R. meliloti* merodiploid strains carrying pRmMK plasmids and of *R. meliloti* homogenotized strains carrying Tn*5*-B20 in the 7.8-kb fragment. **A**, *R. meliloti* merodiploid strains carrying a pRmMK plasmid integrated into megaplasmid 2 result from a single crossing-over (X) between the two copies of the 7.8-kb fragment present on the pRmMK plasmids (▭) and on megaplasmid 2 (▭▭▭). Merodiploid strains are listed. **B**, By double crossing-over (X X), the Tn*5*-B20 transposon (◁) can be homogenotized to the *R. meliloti* genome, in this case to the 7.8-kb fragment of megaplasmid 2. Homogenotized strains obtained are listed. Tc^r = tetracycline-resistance gene of the pRmMK plasmid; Nm^r = neomycin-resistance gene of the transposon Tn*5*-B20.

When the *R. meliloti* merodiploid strains were tested for EPS production and nodule infection, it was found that they behaved like the wild-type *R. meliloti* strain 2011. They produced EPS and formed effective nodules. The only exception was strain Rm2011-pRmMK124. This strain overproduced EPS at the same level as mutant Rm124 and revealed the same symbiotic properties. From this observation it can be concluded that in the merodiploid strain, the gene mutated by Tn*5*-B20 is dominant over the wild-type allele.

The *R. meliloti* mutant Rm154 produces an atypical surface polysaccharide. In contrast to all other described EPS$^-$ mutants, strain Rm154 showed a reduced fluorescence on Cellufluor white agar lacking the typical halo of the wild-type strain (Fig. 4A). We therefore concluded that strain Rm154 produces a different surface polysaccharide and tested whether this polysaccharide can be stained with other dyes. Deep red-colored colonies could be observed when strain Rm154 was grown on minimal agar containing Congo red (Fig. 4B). Because there are reports that cellulose fibrils produced by some *Rhizobium* species give rise to red-colored colonies when stained with Congo red (Kneen and LaRue 1983), we tested Rm154 for the occurrence of these fibrils. Cells of Rm154 were fixed on grids, stained with ruthenium red/uranylacetate, and analyzed by electron microscopy. No microfibrils were observed (data not shown). In addition, strain Rm154 did not show the typical flocculation of cellulose overproducing strains grown in

Table 2. Exopolysaccharide production and symbiotic properties of the homogenotized *Rhizobium meliloti* strains

Strain	Region[a]	Antibiotic[b] marker	EPS[c]	Cfw[d]	Cr[e]	Ab[f]	Nod[g]	Inf[h]	Fix[i]
2011[j]		Sm	100	+	−	−	+	+	+
Rm130	I	Sm,Nm	100	+	−	−	+	+	+
Rm154	II	Sm,Nm	0	(+)	+	+	+	−	−
Rm124	II	Sm,Nm	300	+	−	−	+	+	+
Rm128	II	Sm,Nm	30	+	−	−	+	+	+
Rm110	III	Sm,Nm	0	−	−	−	+	−	−
Rm148	III	Sm,Nm	0	−	−	−	+	−	−
Rm129	III	Sm,Nm	0	−	−	−	+	−	−
Rm113	III	Sm,Nm	0	−	−	−	+	−	−

[a] See Figure 2.
[b] Sm = streptomycin resistance; Nm = neomycin resistance (Tn*5*-B20).
[c] Exopolysaccharide production in %; wild type = 100%.
[d] Fluorescence on Cellufluor white medium when viewed under UV light; + marks fluorescence with halo, (+) marks weak fluorescence without halo (see also Fig. 4A).
[e] Staining behavior on Congo red containing agar; + marks deep red-colored colonies, − marks white or pale pink colonies (see also Fig. 4B).
[f] Staining behavior on aniline blue containing agar; + marks deep blue-colored colonies, − marks white colonies.
[g] Induction of root nodules on alfalfa.
[h] Infection of root nodules.
[i] Nitrogen fixation, measured by acetylene reduction.
[j] *R. meliloti* wild-type strain.

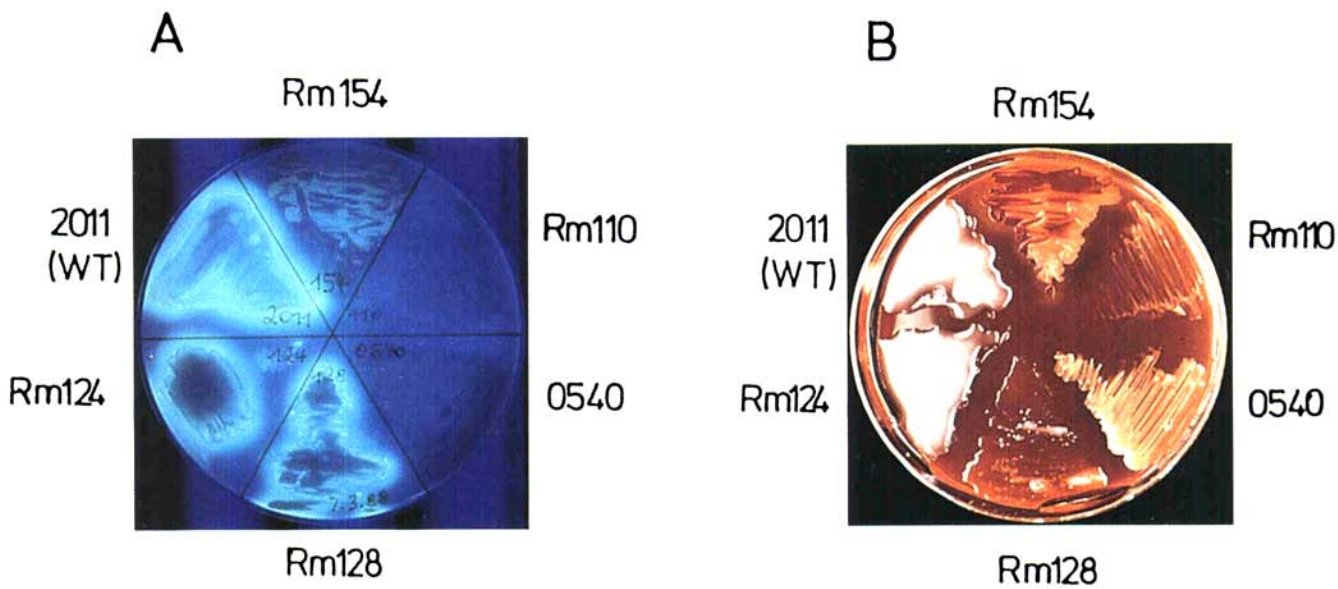


Fig. 4. Staining of *R. meliloti* wild-type and mutant colonies on Cellufluor white and Congo red containing agar. **A**, Strains of *R. meliloti* grown on Cellufluor white agar viewed under UV light. Rm154 shows a slight fluorescence with no halo; Rm110 and mutant 0540 are dark; Rm128, Rm124, and the wild-type 2011 fluoresce brightly. **B**, the same strains as in **A** were grown on Vincent medium containing 0.02% Congo red. Only Rm154 colonies are deep red colored and show uptake of the dye, as could also be seen by the decolorization of the agar. All of the other strains remain white or slightly pink (Rm128).

Table 3. β-galactosidase activity of merodiploid and homogenotized strains carrying Tn*5*-B20 insertions in the 7.8-kb *Eco*RI fragment of megaplasmid 2[a]

Region[b]	Insertion	Merodiploid strain (free living)[c]	Homogenotized strain (free living)[c]	Merodiploid strain (bacteroid)[d]	Homogenotized strain (bacteroid)[d]
I	116	5.0 ± 0.4	...[e]	5.2 ± 0.5	...
	130	13.3 ± 1.4	9.5 ± 8.2	6.1 ± 0.9	7.1 ± 1.2
	120	15.0 ± 1.2	...	7.5 ± 1.4	...
	101	7.3 ± 0.8	...	6.7 ± 1.4	...
	143	6.8 ± 1.2	...	5.9 ± 0.8	...
II	154	21.1 ± 1.9	21.3 ± 4.5	24.2 ± 3.3	n.d.[e]
	124	78.5 ± 8.2	75.1 ± 7.8	94.5 ± 19.5	122.5 ± 8.6
	128	14.0 ± 1.9	15.3 ± 2.7	12.3 ± 4.4	9.2 ± 0.9
III	110	6.4 ± 1.1	14.0 ± 4.1	4.8 ± 1.2	n.d.
	148	8.7 ± 1.2	n.d.	12.3 ± 2.5	n.d.
	113	73.3 ± 4.2	n.d.	39.0 ± 20.0	n.d.
	129	81.4 ± 9.1	n.d.	35.6 ± 8.3	n.d.
IV	119	52.7 ± 3.3	...	11.6 ± 0.7	...
	111	39.1 ± 4.7	...	15.1 ± 1.8	...

[a] The values are calculated from at least six measurements of free-living cells and from three measurements of the bacteroids.
[b] See Figure 2.
[c] Relative β-galactosidase units; background level of *R. meliloti* wild-type strain 2011 was 6.2 ± 0.5.
[d] β-galactosidase units measured for bacteroids; background level of bacteroids of *R. meliloti* wild-type strain 2011 was 2.9 ± 0.8.
[e] ... = no homogenotized strain available; n.d. = not determined.

liquid medium (Zevenhuizen *et al.* 1986). We therefore concluded that Rm154 did not produce cellulose fibrils. Because cells producing the β-(1,3)-glucan curdlan can be specifically stained with anilin blue (Hisamatsu *et al.* 1977), we tested strain Rm154 and other *R. meliloti* strains on anilin blue agar and found that Rm154 formed blue-colored colonies, whereas the colonies of all the other strains remained white (data not shown). We therefore speculate that the *R. meliloti* mutant Rm154 might produce curdlan.

Transcription studies of the 7.8-kb fragment of megaplasmid 2 with *lacZ* as a reporter gene for merodiploid and homogenotized *R. meliloti* strains in both the free-living and bacteroid states. The transposon Tn*5*-B20 used for mutagenesis carries a promoterless *lacZ* gene (Fig. 1), which can also be applied to measure the transcriptional activity at the transposon insertion site. All merodiploid and homogenotized *R. meliloti* strains listed in Figure 3 were used for such studies. The merodiploid strains were considered to be ideal candidates because they show wild-type EPS production and nodule infection. The strains mentioned above were tested for transcriptional activity in both the free-living and the bacteroid states. The results are summarized in Table 3.

The β-galactosidase activities of these strains in the free-living and in the bacteroid state were compared with the background level of the wild-type *R. meliloti* 2011 without Tn*5*-B20 insertion. The background activity resulting from an indigenous *R. meliloti lacZ* gene (Ucker and Signer 1978) was low and did not interfere with the designed experiment. The β-galactosidase activity concerning the merodiploid strains in the free-living state are presented in Figure 5. In this figure, the already defined regions I, II, III, and IV of Figure 1 are indicated again. The transcriptional activity of these four regions will be presented in the following section.

In region I, comprising the Tn*5*-B20 insertions 116, 130, 120, 101, and 143, the measured β-galactosidase activities are close to background level (Fig. 5). Conclusions concerning the transcriptional activities in this region are therefore not possible.

Region II is characterized by the insertions 154, 124, and 128. The transcriptional activity at insertion 124 in free-

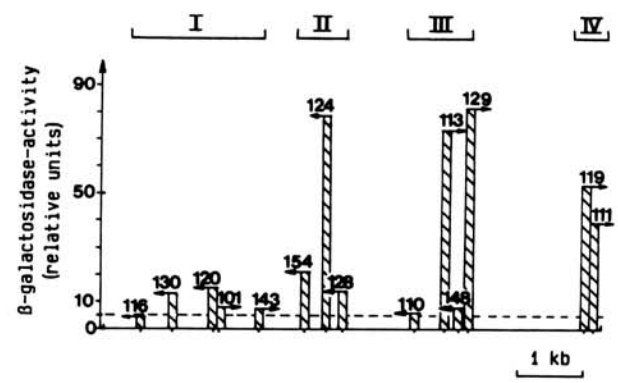


Fig. 5. The transcriptional activities at the Tn*5*-B20 insertion sites in the 7.8-kb *Eco*RI fragment of megaplasmid 2 of *R. meliloti* merodiploid strains in the free-living state are shown. The bars indicate the β-galactosidase activity of the merodiploid strains (relative units). The arrows indicate the transcription orientation of the *lacZ* gene. The dotted line shows the background activity. The regions marked by Roman numbers are as in Figure 2.

living cells is 12 times higher than the background level. Because the *lacZ* genes of the flanking insertions 154 and 128 were expressed at low levels, the transcriptionally active region may include less than 1 kb. Bacteroids of strains Rm124 and Rm2011-pRmMK124 also exhibited a high level of β-galactosidase expression (Table 3). It is therefore concluded that this region is also transcribed in the bacteroid state.

The Tn*5*-B20 insertions 110, 148, 129, and 113 characterize region III (Fig. 5). The *lacZ* gene inserted in the appropriate orientation (insertions 129 and 113) resulted in a 10-fold higher β-galactosidase level than background activity. Insertions in the opposite orientation (110 and 148) gave little or no expression of the inserted *lacZ* gene. This allows us to determine the orientation of transcription of region III, which is from left to right in Figure 5. When isolated bacteroids of nodules induced by strains Rm2011-pRmMK129 and Rm2011-pRmMK113 were tested for their β-galactosidase expression, a relatively high level also could be measured (Table 3). Again, one can assume that in the

bacteroid state, genes located in region III are transcribed.

The Tn*5*-B20 insertions 119 and 111 of region IV exhibited in the free-living and the symbiotic states a remarkably high level of β-galactosidase expression (Fig. 5 and Table 3). But, because no homogenotized strains could be obtained for these two insertions, we were unable to clarify the relevance of this region for EPS production and nodule infection.

DISCUSSION

In this paper we report the Tn*5*-B20 mutagenesis of a 7.8-kb DNA fragment of megaplasmid 2 of *R. meliloti*. This was accomplished by fragment-specific mutagenesis in *E. coli*, with a subsequent conjugational transfer to *R. meliloti*. Two types of transconjugants, merodiploid and homogenotized *R. meliloti* strains, were isolated. Merodiploid strains arise by integration of the 7.8-kb DNA fragment carrying plasmid into the *R. meliloti* genome via single crossing-over. Therefore, the 7.8-kb fragment is duplicated in merodiploid strains. The two copies differ in the insertion of a Tn*5*-B20 transposon that is present in one of the two copies. Homogenotized strains contain only one copy of the 7.8-kb fragment, which carries the Tn*5*-B20 transposon. The experiments showed that the construction of the merodiploid strains was straightforward, whereas homogenotized strains could not be obtained in all cases. For transposon insertions located close to fragment ends, the low probability of a second crossing-over can serve as an explanation. No homogenotization was observed for three insertions in the middle of the 7.8-kb fragment. It might be the case that homogenotization is more difficult for these insertions or that there are viability problems with the homogenotized strains. Altogether, 14 merodiploid and eight homogenotized *R. meliloti* strains were constructed (Figs. 2 and 3).

The merodiploid and homogenotized *R. meliloti* strains were tested for EPS production and nodule invasion. Five out of the eight homogenotized *R. meliloti* strains were symbiotically defective and could no longer carry out the nodule infection step. All of these Inf$^-$ strains were also blocked in the synthesis of an acidic EPS. The three Inf$^-$ strains all produced EPS, but two of them in different amounts when compared with the wild type.

Transposons carrying a *lac*Z gene have been used previously to generate gene fusions and to monitor gene expression at the transposon insertion site. The Mu-d(*lac*) transposons described by Castilho *et al.* (1984), however, have some disadvantages because Mu may cause unspecific mutations in the target DNA (Olson *et al.* 1985). In addition, the missing restriction sites at the ends of the transposons make it difficult to map the transposon insertion site. Other *lac* transposons have been constructed, e.g., Tn*3*-HoHo1 (Stachel *et al.* 1985), Tn*10 lac* (Way *et al.* 1984), and Tn*5 lac* (Kroos and Kaiser 1984). These transposons all carry besides *lac*Z additional parts of the *lac* operon and are therefore difficult to handle. The Tn*5*-B20 transposon used in this study with a size of 8 kb carries only a promoterless *lac*Z gene with ribosomal binding site (Shine-Dalgarno sequence). Therefore, transcriptional fusions are generated at the transposon insertion site.

In this paper we demonstrated that this transposon is an extremely useful tool for transcriptional studies. In particular, merodiploid strains that behave like wild type concerning EPS production and nodule infection are ideal constructions for such studies, because transcriptional activity can be measured in those strains without influencing the normal phenotype. This is especially the case for Inf$^-$ mutants, which do not infect nodules and hence are unable to form bacteroids. In addition, integration of the pRmMK plasmids into the genome gives a ratio of 1:1 concerning the wild-type and the Tn*5*-B20 mutated gene. The most interesting question of this study is of course whether the transcriptional activity of genes involved in EPS production and nodule infection is blocked in the symbiotic state. Therefore, we also analyzed bacteroids isolated from alfalfa nodules and found, in general, that all those inserted *lac* genes exhibited a remarkably high level of transcription which were efficiently expressed in the free-living state.

According to the location of the different Tn*5*-B20 insertions, we defined the regions I, II, III, and IV in the 7.8-kb DNA fragment, as indicated in Figure 2. These regions will now be discussed in detail.

Region I. The Tn*5* mutant 2505 (Müller *et al.* 1988), defining this region, produces no EPS and is defective in nodule invasion. The Tn*5*-B20 insertions surrounding the mutation 2505 could not be homogenotized. Therefore, we do not know at present whether other genes located in this region are involved in EPS production and nodule infection.

Region II. This region carrying mutation 154 is evidently essential for acidic EPS synthesis and nodule infection. The other two insertions of this region revealed different phenotypes. Two strains (Rm124 and Rm128) exhibited no symbiotic defects but were affected in EPS production. Mutation 124 is the first one on megaplasmid 2 that is responsible for the production of more EPS than the wild type. Other EPS overproducing mutants of *R. meliloti* described until now carried mutations in chromosomal genes (Müller *et al.* 1988; G. C. Walker, personal communication). Strain Rm128 showed a reduced level of EPS production. The already mentioned insertion 154 of region II strengthened the hypothesis that EPS is involved in nodule infection. Although Rm154 produced no EPS, the colonies of Rm154 showed a reduced fluorescence with no halo on Cellufluor white plates. Leigh *et al.* (1987) reported a similar phenotype for *exo*H mutants of *R. meliloti*, which, however, still produced EPS. This EPS was structurally altered, lacking the succinate residue that Leigh and coworkers thought to be responsible for the altered staining behavior and the infection defects. The *exo*H gene is located approximately 6.5 kb apart from region II (G. C. Walker, personal communication). When stained with Congo red and anilin blue, strain Rm154 revealed dark-colored colonies. Cellulose fibrils could be stained with Congo red, but because no fibrils could be observed by electron microscopy and because aniline blue stains the β-(1,3)-glucan curdlan very specifically (Hisamatsu *et al.* 1977), it can be speculated that Rm154 produces curdlan. *Agrobacterium* strains are known to produce curdlan (Hisamatsu *et al.* 1977), but for *R. meliloti* SU47 (the parent strain of *R. meliloti* 2011) and other *R. meliloti* strains, curdlan could not be detected (Zevenhuizen *et al.* 1986; Ghai *et al.* 1981). More specific tests are now necessary to identify the chemical nature of this hypothetical polysaccharide. Transcription studies of region II revealed that the central area of this region is expressed at a high level in the free-

living and in the symbiotic state. This means that no transcriptional repression occurs in the bacteroid state.

Region III. This region (Fig. 2) has been originally identified by the Tn*5* mutant 0540 (Müller *et al.* 1988). The Tn*5*-B20 insertions 110, 148, 129, and 113 extended this region to span at least one kilobase. Region III corresponds putatively to the *exo*F locus previously described by Leigh and coworkers (1985) as concluded by comparing restriction maps (G. C. Walker, personal communication). The transcription direction of region III could be deduced by the four Tn*5*-B20 insertions (Fig. 5) and confirmed the data of Walker and coworkers (personal communication), who used Tn*phoA* (Manoil and Beckwith 1985) for determining the transcriptional direction. Transcription studies concerning the insertions 113 and 129 were also carried out for bacteroids. The data presented in Table 2 revealed that no transcriptional repression could be observed in the bacteroid state.

Region IV. The two Tn*5*-B20 insertions on the right end of the 7.8-kb DNA fragment (Fig. 2) are located in region IV. No mutants were obtained for these insertions. The relevance for EPS production and symbiosis has still to be examined as the high expression level of the introduced *lacZ* gene of the merodiploid strains revealed that region IV is transcriptionally active in the free-living and in the bacteroid state.

The results presented here, together with those by Müller *et al.* (1988), demonstrate that the 7.8-kb fragment of the second megaplasmid of *R. meliloti* carries at least three regions (I, II, and III) involved in EPS synthesis and in nodule infection. For two regions (II and III), it was found that EPS and infection genes were actively transcribed during symbiosis. Further investigations are necessary to determine the role of these genes in *R. meliloti* bacteroids.

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