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Molecular Analysis of the *Rhizobium meliloti* *mucR* Gene Regulating the Biosynthesis of the Exopolysaccharides Succinoglycan and Galactoglucan

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The *Rhizobium meliloti* Tn*5* mutant Rm3131, producing galactoglucan (EPS II) instead of succinoglycan (EPS I), was complemented by a 3.6-kb *Eco*RI-fragment of the *Rhizobium meliloti* genome. Sequencing of this fragment revealed six open reading frames (ORFs). The ORF found to be affected in the mutant Rm3131 codes for a putative protein of 15.7 kDa and forms a monocistronic transcriptional unit. Further genetic analysis revealed that the gene mutated in Rm3131 is identical to the previously described *R. meliloti mucR* gene (H. Zhan, S. B. Levery, C. C. Lee, and J. A. Leigh, 1989, Proc. Natl. Acad. Sci. USA 86:3055-3059). By hybridization it was shown that a *mucR* homologous gene is present in several rhizobacteria. The deduced amino acid sequence of MucR showed nearly 80% identity to the *Agrobacterium tumefaciens* Ros protein, a negative regulator of *vir* genes and necessary for succinoglycan production. MucR contains like Ros a putative zinc finger sequence of the C_2H_2 type. Transcriptional fusions of genes for EPS I and EPS II synthesis, the so-called *exo* and *exp* genes, with the marker gene *lacZ* were used to delineate the role of *mucR* for *exo* and *exp* gene expression. It was found that *exp* genes are negatively regulated by MucR on the transcriptional level, whereas a posttranscriptional regulation by MucR is assumed for *exo* genes. Furthermore, *mucR* is negatively regulating its own transcription.

Additional keywords: exopolysaccharide synthesis, gene regulation, symbiosis.

This paper is dedicated to Prof. Dr. P. Sitte on the occasion of his 65th birthday.

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The formation of nitrogen-fixing root nodules is the result of a complex interaction between leguminous plants and soil bacteria of the genus *Rhizobium* (Djordjevic et al. 1987; Long 1989). For various *Rhizobium* species it was found that exopolysaccharides play an important role in this interaction (Leigh and Coplin 1992). *Rhizobium meliloti* produces the acidic exopolysaccharide EPS I, also designated succinoglycan (Aman et al. 1981). Mutants of *R. meliloti* lacking EPS I are characterized by defects in nodule infection (Finan et al. 1985, Leigh et al. 1985, Müller et al. 1988). A signal molecule of the EPS I biosynthetic pathway is thought to be necessary for the recognition of *R. meliloti* by its host plant (Battisti et al. 1992). For EPS I biosynthesis 19 *exo* genes comprising a 24-kb DNA region of the megaplasmid 2 have recently been sequenced (Buendia et al. 1991; Reed et al. 1991a; Müller et al. 1993; Becker et al. 1993a, 1993b, 1993c; Glucksmann et al. 1993a, 1993b).

In addition, *R. meliloti* Rm2011 has the cryptic ability to synthesize the alternative exopolysaccharide EPS II also designated galactoglucan (Her et al. 1990; Levery et al. 1991); *R. meliloti* strains containing a mutation in the chromosomally located genes *expR* (Glazebrook and Walker 1989) or *mucR* (Zhan et al. 1989) produce EPS II instead of EPS I. Therefore, these genes were assumed to be regulators of both, EPS I and EPS II synthesis (Leigh et al. 1993; Gonzalez et al. 1993). The *exp* genes responsible for EPS II synthesis have been mapped on a 23-kb DNA region of the megaplasmid 2. They are organized in six complementation groups (Glazebrook and Walker 1989). Several other genes involved in EPS biosynthesis and regulation, like *exoC*, *exoR*, and *exoS*, have been identified on the chromosome (Finan *et al.* 1986; Doherty et al. 1988; Reed et al. 1991b).

In the present study, we describe the *R. meliloti* Tn*5* mutant Rm3131, which does not produce detectable amounts of the EPS I polymer, but synthesizes significant amounts of the galactoglucan EPS II. We report the identification and sequencing of a DNA fragment complementing the defects of mutant Rm3131. Further characterization resulted in the identification of the *mucR* gene mutated in Rm3131. We focused on the regulation of the gene itself and its influence on *exo* and *exp* gene expression.

Parts of this work were presented at the Fifth International Symposium on Plant-Microbe Interactions, Interlaken, Switzerland, 1990, and the Ninth International Symposium on Nitrogen Fixation, Cancun, Mexico, 1993 (Pühler et al. 1991, 1993).

RESULTS

The symbiotically efficient *R. meliloti* mutant Rm3131 produces galactoglucan (EPS II) and only traces of succinoglycan (EPS I).

Following a general Tn*5* mutagenesis of the *R. meliloti* wild-type strain Rm2011 we isolated mutant Rm3131 due to its altered colony morphology. When grown on Calcofluor white agar, an indicator medium for succinoglycan production in *R. meliloti* (Finan et al. 1985), despite their mucoid appearance Rm3131 colonies showed only a dim UV-fluorescence with a weak fluorescing halo in contrast to the bright fluorescence of the wild-type Rm2011 (Fig. 1, Table 1). This may indicate the production of very small amounts of a low molecular weight EPS I in addition to a further exopolysaccharide. We transduced the *exoY*::Tn*5*-Gm mutation of the EPS I$^-$ mutant RmJQ13 (Kapp et al. 1990) into mutant Rm3131 and found that the resultant double mutant RmJQ46 showed no UV fluorescence with Calcofluor (Table 1) and was therefore no longer able to produce EPS I. Nevertheless, RmJQ46 like Rm3131 formed mucoid colonies due to the production of the second exopolysaccharide.

We isolated this exopolysaccharide from the supernatants of Rm3131 and RmJQ46 cultures. ^{13}C and ^{1}H nuclear magnetic resonance (NMR) analyses of the exopolysaccharides isolated from Rm3131 and RmJQ46 showed that the two mutants synthesized the same exopolysaccharide (data not shown) which differed significantly from the wild-type EPS. The ^{13}C spectra of the exopolysaccharide from the wild-type Rm2011 and the double mutant RmJQ46 are shown in Figure 2. Interpretation of the ^{13}C-NMR spectra led to the conclusion that mutant Rm3131 produced the galactoglucan (EPS II) described by Her et al. (1990) and Levery et al. (1991) instead of succinoglycan (EPS I). This conclusion could be confirmed by determining the ratio of glucose to galactose in EPS I and EPS II. As expected EPS I showed a ratio of 7:1, whereas EPS II showed a ratio of 1:1 (data not shown).

Since the mutation of strain Rm3131 caused both, the production of EPS II and a very strong reduction in EPS I biosynthesis, we propose that the gene mutated in Rm3131 is involved in the regulation of EPS I and EPS II biosynthesis.

EPS I is known to be essential for the infection of alfalfa nodules. Therefore, we analyzed the symbiotic properties of the mutants Rm3131 and RmJQ46. Rm3131 was able to invade alfalfa nodules and 3 wk after inoculation induced a normal, nitrogen-fixing symbiosis (Table 1). In contrast, the double mutant RmJQ46 (EPS I$^-$) induced predominantly empty nodules (pseudonodules) (Table 1). Only very few nodules were found to be infected 3 wk after inoculation but no nitrogen was fixed by these nodules.

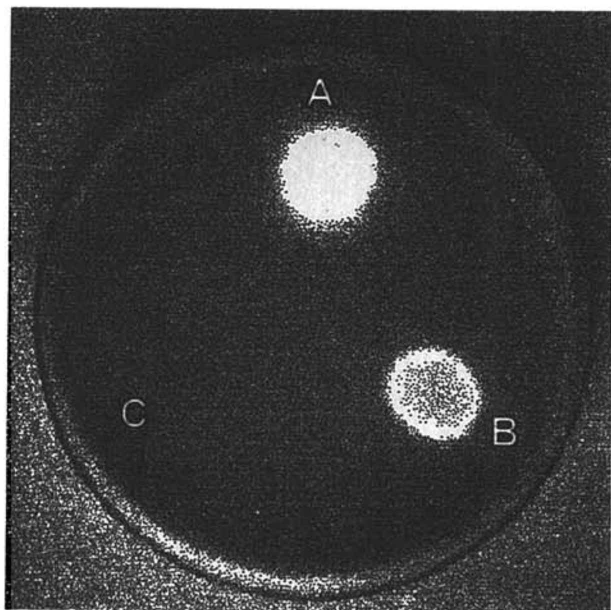

Fig. 1. Ultraviolet fluorescence of different *Rhizobium meliloti* strains grown on minimal agar containing Calcofluor white. **A,** The *R. meliloti* wild-type strain Rm2011 fluoresces brightly. **B,** The *R. meliloti* mutant strain Rm3131 shows only a dim fluorescence with a weak fluorescing halo. **C,** The *R. meliloti* EPS I$^-$ mutant strain RmJQ13 shows no fluorescence and appears Calcofluor dark.

The *R. meliloti* mutant Rm3131 is mutated in *mucR*.

To identify the affected gene, mutant Rm3131 was complemented using a cosmid gene bank. Two complementing cosmids (cJQ1 and cJQ2) were isolated and physically mapped (Fig. 3A). The cosmids span a DNA region of 59.8 kb and showed an overlap of 15.8 kb. A 3.6-kb *Eco*RI subfragment common to both cosmids was cloned, resulting in plasmid pMS136 which was able to complement mutant Rm3131. By in vivo cloning, using plasmid pMS136, we isolated the *Eco*RI fragment carrying the Tn*5* insertion of Rm3131. Restriction analysis revealed the Tn*5* insertion of Rm3131 to be located on the 3.6-kb *Eco*RI fragment (Fig. 3B). By hybridization we could assign this fragment to the chromosome (data not shown).

Since the observed phenotype of mutant Rm3131 was similar to that of the previously described *R. meliloti mucR* mutant Rm7010 (Zhan et al. 1989) we transferred plasmid pMS136 into strain Rm7010. This plasmid was able to complement the defects of the *mucR* mutant Rm7010. Further-

Table 1. Properties of the *Rhizobium meliloti* wild type and exopolysaccharide mutants

Strain	Genotype	Colony	Cfw[a]	EPS type[b]	Nodule type[c]
Rm2011	wt	Mucoid	Bright	I	wt
Rm3131	*mucR*[d]	Mucoid	Dim + halo	II/I[tr]	wt
RmJQ13	*exoY*	Rough	Dark	–	Pseudo
RmJQ46	*mucR, exoY*	Mucoid	Dark	II	Pseudo[e]

[a] UV fluorescence of colonies in the presence of the dye Calcofluor white.

[b] The EPS type produced was determined by the glucose: galactose ratio and by ^{1}H and ^{13}C NMR spectroscopy, tr: traces of EPS I.

[c] Nodule structure 3 wk after inoculation. wt: pink, cylindrical nodule; pseudo: white, spheroid nodule without bacteroids.

[d] The gene affected in Rm3131 will be shown later in this article to be *mucR*.

[e] In addition to pseudonodules a few nodules were infected, but these nodules remained non-fixing.

more, hybridization of *Eco*RI-digested total DNA of mutant Rm7010 with plasmid pMS136 revealed that the transposon insertion of this mutant is located on the 3.6-kb *Eco*RI fragment (data not shown). Complementation was also obtained with plasmid pAR300, containing a 1.2-kb *Sph*I–*Xho*I subfragment of plasmid pMS136. As will be shown below, only one complete open reading frame (ORF) is located on this fragment. We have previously termed the gene affected in Rm3131 as *rexA* (Keller et al. 1990). The results shown above revealed that the insertions of the *rexA* mutant Rm3131 and of the *mucR* mutant Rm7010 are localized in the same ORF. Therefore, we adopted the nomenclature of Zhan et al. (1989) and now also term the gene mutated in Rm3131 *mucR*.

Sequence analysis of a 3.6-kb *Eco*RI fragment complementing the *R. meliloti* mutant Rm3131 resulted in the identification of the *mucR* coding region.

To further characterize the gene mutated in Rm3131 we sequenced the isolated 3.6-kb *Eco*RI fragment of plasmid pMS136. By using the computer program Analyseq (Staden 1986) six open reading frames (ORFs) could be identified on the 3.6-kb *Eco*RI fragment (Fig. 3B). ORF 1, located at the left end, and ORF 6, located at the right end of the sequenced fragment are incomplete.

Sequencing of the Tn*5* insertion of mutant Rm3131 revealed its location in ORF 3, which was designated *mucR* (see above). The nucleotide and deduced amino acid sequence of the 1.2-kb *Sph*I–*Xho*I fragment containing *mucR* is presented in Figure 4. The *mucR* coding region starts at nucleotide 328 and ends at nucleotide 757 of the *Sph*I–*Xho*I fragment. The ATG start codon is preceded at a reasonable distance by a potential ribosomal binding site. Upstream of the *mucR* coding region we identified sequences with homology to the consensus sequence of the *E. coli* σ^{70} promoter with four and three out of six matches with the –35 and the –10 region of the consensus sequence, respectively (McClure 1985). Downstream of the *mucR* coding region an inverted repeat could be located, probably forming a stem-loop structure with a Gibbs free energy (ΔG) of –128 kJ/mol (Tinoco et al. 1973) which may function as a transcriptional terminator.

Genes homologous to *R. meliloti mucR* are also present in other bacteria.

The *R. meliloti mucR* gene mutated in strain Rm3131 encodes a protein of 15.7 kDa. A comparison of the amino acid sequence of MucR to sequences of the EMBL database revealed a significant homology to the Ros protein of *Agrobacterium tumefaciens* (Cooley et al. 1991; Fig. 5A). The Ros protein of *A. tumefaciens* was identified as a negative regulator of the *vir* genes and a positive regulator of the succinoglycan synthesis. Like Ros, the *mucR*-encoded protein contains a potential zinc finger motif of the C_2H_2 type (Berg 1990) (Fig. 5A). Upstream of the *mucR* coding region a sequence with homology to the Ros box (Cooley *et al.* 1991) could be identified (Fig. 5B). No significant homology of the deduced amino acid sequence of ORF 1, 2, and 4 to 6 to sequences of the EMBL database could be found.

To analyze whether genes homologous to *mucR* are present in other bacteria, we hybridized the 1.2-kb *Sph*I–*Xho*I fragment carrying *mucR* with *Eco*RI digested total DNA of vari-

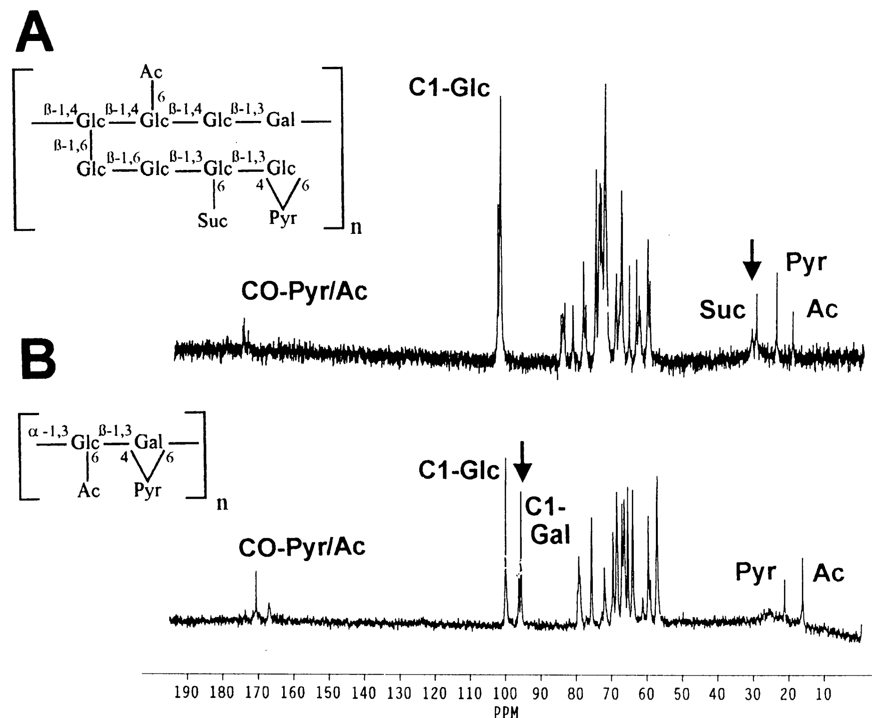

Fig. 2. ^{13}C-NMR spectra and structure of exopolysaccharides produced by the *Rhizobium meliloti* wild-type strain Rm2011 **(A)** and the *R. meliloti* mutant RmJQ46 **(B)**. The following signals of the spectra could be identified. Ac: Signal from the methyl group of acetate; Pyr: signal from the methyl group of pyruvate; Suc: signal from the methyl group of succinate; C1-Gal: signal from the glycosidic linked C1 of galactose. C1-Glc: signal from the glycosidic linked C1 of glucose; CO-Pyr/Ac: signal from the carboxy group of acetate and pyruvate. The arrows indicate the most striking differences between the two spectra: the methyl groups of the succinyl residue of the EPS I and the glycosidic bound C1 of the galactose in the EPS II spectrum.

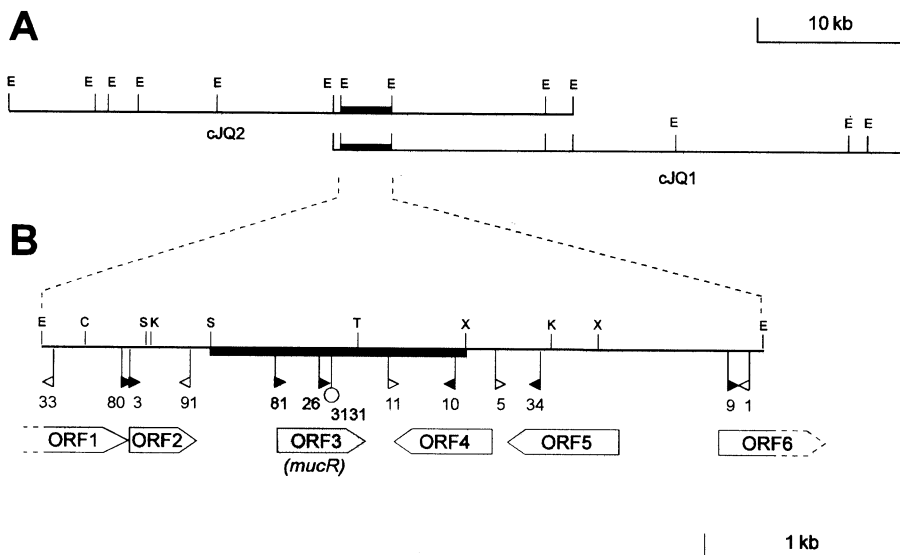

Fig. 3. Organization of the chromosomal *Rhizobium meliloti* DNA region carrying the *mucR* gene regulating exopolysaccharide synthesis. **A,** Map of a 59.8-kb DNA region represented by the inserts of the two cosmid clones cJQ1 and cJQ2. The 3.6-kb *Eco*RI fragment complementing mutant Rm3131 is indicated by a black bar. **B,** Detailed map of the 3.6-kb *Eco*RI fragment. A 1.2-kb *Sph*I-*Xho*I subfragment still complementing mutant Rm3131 is marked by a black bar. The extension of the identified six open reading frames (ORFs) is given. The Tn*5*-B20 insertions are shown as flags indicating the transcription direction of the promotorless *lacZ* gene in the Tn*5*-B20 transposon. Black triangles indicate the expression of the *lacZ* gene. The Tn*5*-B20 insertions are numbered. The pin shows the Tn*5* insertion site of mutant Rm3131. Abbreviations: E, *Eco*RI; C, *Cla*I; X, *Xho*I; K, *Kpn*I; S, *Sph*I; T, *Tth*111I.

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GCATGCCGTTAAATTTATGTTTGGACTTTTGGCGGATCAGTGTTAATGCAGTAAATGCAAAGTGTCGTTGTGGAAAATACTTTGAACTGA
      10        20        30        40        50        60        70        80        90

AATTCCAGCCTGAGTTGTGATGCTTGTCCGTTGCTTTGAGGTGTCGGCCGCTTTGGCGCCTTGGTGACGGATCTTCCCTCTCGCAATTCG
     100       110       120       130       140       150       160       170       180

AGCCTTGATGTCGCGGCAGAACCGCAGTCTCCATCGCGAGATAGCGGTCGGTTCCGAGGCGGCAAGCGCTAATCGACGGCATTTTGTACA
     190       200       210       220       230       240       250       260       270

                                                         M  T  E  T  S  L  G  T  S  N  E
TCCTCCCCCTGAAACTCTGTTTCAGGTTTCGTCGCTGGATTCCGATAGGAGAAAGAAATGACAGAGACTTCGCTCGGTACGAGCAATGAA
     280       290       300       310       320       330       340       350       360

 L  L  V  E  L  T  A  E  I  V  A  A  Y  V  S  N  H  V  V  P  V  A  E  L  P  T  L  I  A  D
CTCCTGGTTGAGCTTACGGCGGAGATCGTTGCCGCCTATGTAAGCAACCACGTGGTTCCGGTTGCCGAGCTGCCGACGCTCATAGCCGAC
     370       380       390       400       410       420       430       440       450

 V  H  S  A  L  N  N  T  T  A  P  A  P  V  V  V  P  V  E  K  P  K  P  A  V  S  V  R  K  S
GTGCATTCGGCTCTCAACAACACCACGGCTCCTGCGCCGGTGGTCGTCCCGGTGGAAAAGCCGAAGCCTGCGGTTTCGGTCCGAAAGTCG
     460       470       480       490       500       510       520       530       540

 V  Q  D  D  Q  I  T  C  L  E  C  G  G  T  F  K  S  L  K  R  H  L  M  T  H  H  N  L  S  P
GTGCAGGACGACCAGATCACATGTCTCGAATGCGGCGGCACCTTCAAGTCGTTGAAGCGGCATCTGATGACGCACCACAACCTTTCGCCG
     550       560       570       580       590       600       610       620       630

 E  E  Y  R  D  K  W  D  L  P  A  D  Y  P  M  V  A  P  A  Y  A  E  A  R  S  R  L  A  K  E
GAAGAATACCGCGACAAGTGGGATTTGCCTGCGGATTATCCGATGGTCGCACCGGCCTACGCGGAAGCGCGCTCGCGGCTCGCCAAGGAA
     640       650       660       670       680       690       700       710       720

 M  G  L  G  Q  R  R  K  R  R  G  K  *
ATGGGTCTCGGACAGCGTCGGAAGCGTCGCGGCAAGTGATCCGGGCGGCCCCACTGGGCTCGGGAACGCGCAAGCGAACACCTGAGTTGT
     730       740       750       760       770       780       790       800       810

GTCATAAAAGCCGGCCGGTTCAGGCCGGCTTTTTTTCGACGTTCAGTATTTCATTGAAACACTGACACACTCTAACCCTTCGAGGCTACG
     820       830       840       850       860       870       880       890       900

CACTTCCGGACGGAAAACCGTTACGCACCTTTCCTCGAAGTGCTCTAACTCTTTGCCAGTCAGGCGGGCAGGCGCCTTCGGCTTCGCTCT
     910       920       930       940       950       960       970       980       990

TGATACGCCGGATCATCGAGCGCAGGCCGTTCGCGCGTTGCGAGGAAAGATGTTCGATGAGGCCGATCTTGCCGAAGATGTCGAGAGCGT
    1000      1010      1020      1030      1040      1050      1060      1070      1080

CGATTCTGGTAATTTCCGAAGCACGCTTACCGGAGAAGATCGCGAGGACGATCGCCACGAGGCCGCGCACGATATGCGCATCCGATTCTC
    1090      1100      1110      1120      1130      1140      1150      1160      1170

CCTCGAAGGTGAGGAGCGGGTCTTCAGCATCGCCGGTCGCATGGGTCACGAGCCAGACCTGGCTGGCACAGCCTTGAACCTTATTCTCGC
    1180      1190      1200      1210      1220      1230      1240      1250      1260

TCGTCCTCGAG
    1270
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ORF 3 (*mucR*)

Fig. 4. Nucleotide sequence of the 1.2-kb *Sph*I-*Xho*I fragment complementing mutant Rm3131. The DNA sequence is presented in 5′-3′ direction. The deduced amino acid sequence of the identified ORF 3 (*mucR*) is given in the single letter code. The pin marks the transposon insertion site of mutant Rm3131. A potential transcriptional terminator (inverted repeat) downstream of ORF 3 (*mucR*) is shown by arrows, a putative *mucR* binding site (inverted repeat) upstream of ORF 3 (*mucR*) is underlined. A potential ribosomal binding site is indicated by overscoring. The sequence homologous to the *E. coli* σ^{70} promoter consensus sequence upstream of ORF 3 (*mucR*) is boxed.

ous bacterial species. For several rhizobacteria like *A. tumefaciens*, *Rhizobium* sp. strain NGR234, *R. fredii*, *R. leguminosarum* bv. *phaseoli*, *Pseudomonas fluorescens,* and *Erwinia stewartii* a hybridizing band was found that varied in size in the different bacteria (data not shown). Surprisingly, no hybridization was detected with total DNA of *R. l.* bv. *trifolii* and *R. l.* bv. *viciae*, even at low stringency conditions.

The *mucR* gene forms a monocistronic transcription unit.

To obtain information about the function of ORF 1, 2, and 4 to 6 we carried out a mutational analysis of the 3.6-kb *Eco*RI fragment. Plasmid pMS136 carrying the 3.6-kb *Eco*RI fragment was mutagenized in *E. coli* using the *lacZ* transposon Tn*5*-B20 (Simon et al. 1989). Insertions in all ORFs could be isolated (Fig. 3B). The insertion sites of the Tn*5*-B20-transposons were determined by sequencing (data not shown). The Tn*5*-B20 insertions were subsequently homogenotized into the *R. meliloti* wild-type strain Rm2011. The resulting mutants showed significant β-galactosidase activities when the *lacZ* gene had the same transcriptional direction as the respective ORF, indicating that these ORFs may represent genes. All Tn*5*-B20 mutants were further analyzed to determine their symbiotic properties and EPS production. With the exception of the *mucR* mutants Rm26 and Rm81 (Tn*5*-B20 insertions 26 and 81 in pMS136, Fig. 3B), no altered phenotype could be detected. Therefore, the functions of the remaining ORFs are still unknown.

The *mucR* mutants Rm26 and Rm81 showed the same phenotype as the original mutant Rm3131. We propose that *mucR* forms a monocistronic transcription unit because mutations in ORF 2 had no effect on EPS production and ORF 4 reads opposite to ORF 3.

MucR represses the transcription of *exp* genes directing the synthesis of the exopolysaccharide EPS II.

Since the *R. meliloti mucR* mutant Rm3131 produced EPS II we supposed that *mucR* may repress the biosynthesis of this exopolysaccharide. Therefore, we studied the influence of MucR on the expression of *exp* genes responsible for EPS II biosynthesis. Using the *expA* mutant Rm8605 (Glazebrook and Walker 1989; Fig. 6) we isolated a 8.9-kb *Kpn*I DNA fragment carrying almost the entire *expA* complementation group of *R. meliloti* Rm2011 (for details see Materials and Methods). The 8.9-kb *Kpn*I fragment and a 3-kb *Bgl*II-*Kpn*I subfragment were cloned into plasmid pK18*mob,* resulting in plasmids pAR1 and pAR1-2, respectively. For the construction of *exp-lacZ* transcription fusions, a promoterless *lacZ*-Gm^r interposon was cloned into the single *Bgl*II site of plasmid pAR1 and, in both orientations, into the single *Hin*dIII site of plasmid pAR1-2. The resulting *expA–lacZ* fusions were introduced by marker exchange into the wild-type strain Rm2011 and into the *mucR* mutant Rm3131, resulting in the *R. meliloti* strains RmAR1011 to RmAR1013 and RmAR1111 to RmAR1113, respectively. In the absence of a functional *mucR* gene the expression of the *exp–lacZ* fusions in strains RmAR1111 and RmAR1113 were at least 16-fold higher than in the presence of a functional *mucR* gene in the strains RmAR1011 and RmAR1013 (Fig. 6). Therefore, it is proposed that MucR negatively regulates the transcription of at least some of the *exp* genes located within the *expA* complementation group. The control strains RmAR1012 (MucR$^+$) and RmAR1112 (MucR$^-$) with the *lacZ* gene inserted into the *expA* region in opposite orientation showed only background expression.

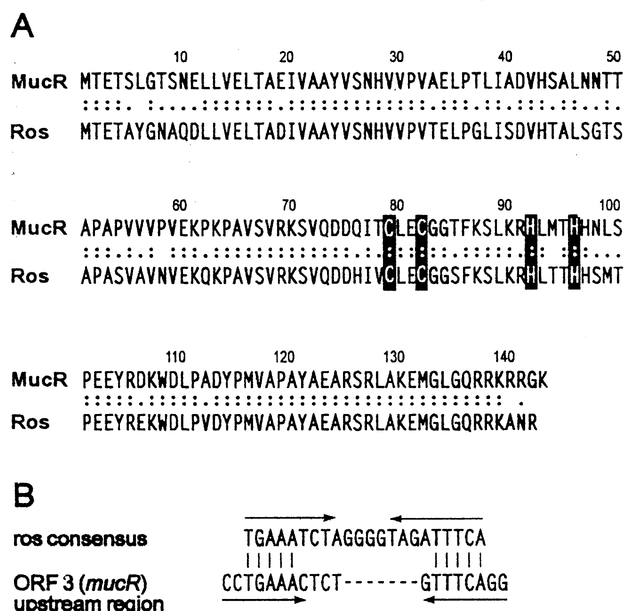

Fig. 5. Comparison of the *Rhizobium meliloti* MucR protein with the *Agrobacterium tumefaciens* Ros protein. **A,** Comparison of the deduced amino acid sequences of MucR and Ros. Identical amino acids are marked by colons and homologous amino acids by a fullstop. Both proteins share 78.3% identical amino acids. The amino acids which constitute the potential zinc finger motif in both proteins, are shadowed. **B,** Comparison of the putative binding site of MucR to the *ros* consensus sequence. The inverted repeats are marked by arrows.

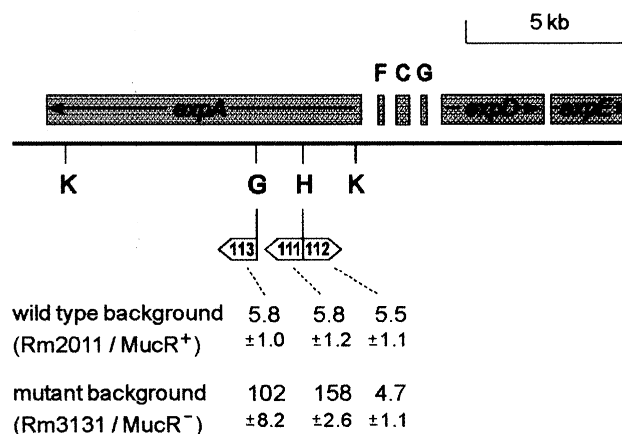

Fig. 6. Expression of *exp* genes in a MucR$^+$ and a MucR$^-$ background. The restriction map and the position of the complementation groups of the *exp* region, according to Glazebrook and Walker (1989), are presented. The *lacZ*-Gm^r interposon insertion sites are shown with arrows indicating the transcriptional orientation of the *lacZ* gene. The numbers indicate the corresponding insertions in *Rhizobium meliloti* strains RmAR1011 to RmAR1013 and RmAR1111 to RmAR1113, respectively. The β-galactosidase activities as well as the standard deviations for the wild type and the MucR$^-$ background are given. Controls without *lacZ*-Gm^r interposon: Rm2011, 3.9 ± 1.7 and Rm3131, 1.1 ± 0.9. Abbreviations: G, *Bgl*II; H, *Hin*dIII; K, *Kpn*II.

MucR does not markedly influence the transcription of *exo* genes directing the synthesis of the exopolysaccharide EPS I.

As shown for the *R. meliloti* mutant Rm3131, the *mucR* mutation causes a drastic reduction of EPS I production. To study the expression of *exo* genes in a wild type and a *mucR* mutant background, we used or constructed *R. meliloti* Rm2011 and Rm3131 derivatives containing *lacZ* transcription fusions to *exoP*, *exoN*, *exoO*, *exoA*, *exoK*, *exoH*, *exoT*, *exoY*, and *exoF* (for details see Table 1, Fig. 7, and the Materials and Methods section). Only the *exoF–lacZ* and *exoY–lacZ* fusions showed a slightly (25–33%) reduced activity in the *mucR* mutant background (strains RmAR9017 and RmAR9018) when compared to the wild-type background (strains RmAR9007 and RmAR9008). The genes *exoF* and *exoY* belong to the same transcription unit (Müller et al. 1993). The activity of the *exoK–lacZ* fusion was reduced by ~66% in the *mucR* mutant background (strain RmAR9011 compared to strain RmH2a). In all the other *R. meliloti* strains carrying *exo–lacZ* fusions, no influence by *mucR* could be observed (Fig. 7).

MucR negatively regulates its own transcription.

When we compared the homogenotized *mucR–lacZ* mutants Rm26 and Rm81 (Fig. 3B) to the merodiploid strains Rm2011-pAR26 and Rm2011-pAR81 carrying a *mucR–lacZ* fusion and a functional *mucR* gene we measured fivefold higher β-galactosidase activities for the homogenotized strains (Fig. 8, lines 1 + 2). From these data we assumed that MucR may negatively regulate its own transcription. Therefore, we further studied the possible autoregulation of *mucR* in more detail. To rule out that the *mucR* expression in strains Rm2011-pAR26 and Rm2011-pAR81 is influenced by titrating out a potential activator protein due to an additional copy of the *mucR* gene region, we cloned the 3.6-kb *Eco*RI fragment into the broad host range vector pSUP104 replicating with approximately 100 copies in *R. meliloti* (Labes *et al.* 1990). The resulting plasmid was termed pAR360. Additionally, we constructed plasmid pAR361 by filling in the *Tth*111I site of plasmid pAR360 which resulted in a frameshift mutation in the *mucR* coding region. Additional copies of *mucR* in strains Rm26 and Rm81 achieved by introduction of plasmid pAR360 resulted in a tenfold reduction of the expression of the *mucR–lacZ* fusion compared to strains Rm26 and Rm81 carrying plasmid pAR361 (Fig. 8, lines 3 + 4). Additionally, Figure 8 shows that a high copy number of the *mucR* gene (plasmid pAR360 in strains Rm26 and Rm81) caused a twofold repression of the *mucR-lacZ* fusion compared to a single copy of *mucR* in strains Rm2011-pAR26 and Rm2011-pAR81. Therefore, we concluded that MucR may be a negative regulator of its own transcription. To analyze a possible direct influence of *mucR* on its own transcription, we performed additional experiments in an *E. coli* background in the presence or absence of a functional *mucR* gene. We transformed plasmid pAR300 carrying a subcloned intact *mucR* gene, and the compatible plasmid pAR26 carrying the *mucR–lacZ* fusion, into *E. coli* MC1000. The *E. coli* MC1000 strain harboring only plasmid pAR26 carried no intact *mucR* gene. The *mucR–lacZ* fusion was expressed at a medium level (Fig. 8, line 5). When plasmid pAR300 with a functional *mucR* gene was also transferred to this strain, we observed, as in *R. meliloti,* a decrease in the expression of the *mucR-lacZ* fusion (Fig. 8, line 6). The decrease in *lacZ* expression was, however, only twofold.

DISCUSSION

In this paper we report the characterization of the pleiotropic *R. meliloti* mutant Rm3131 and the gene affected in this mutant. We found that Rm3131 mainly synthesized galactoglucan (EPS II) as was verified by NMR spectroscopy and analysis of the glucose-galactose ratio of this exopolysaccharide. Additionally, Rm3131 produced traces of the wild-type exopolysaccaride succinoglycan (EPS I) which account for the Calcofluor dim phenotype and the weak fluorescing halo around Rm3131 colonies. This finding could be confirmed by transduction of an *exoY* mutation into Rm3131. The resultant double mutant RmJQ46 produced only EPS II and was no longer stainable with Calcofluor white. When we analyzed the symbiotic properties of the EPS II producing

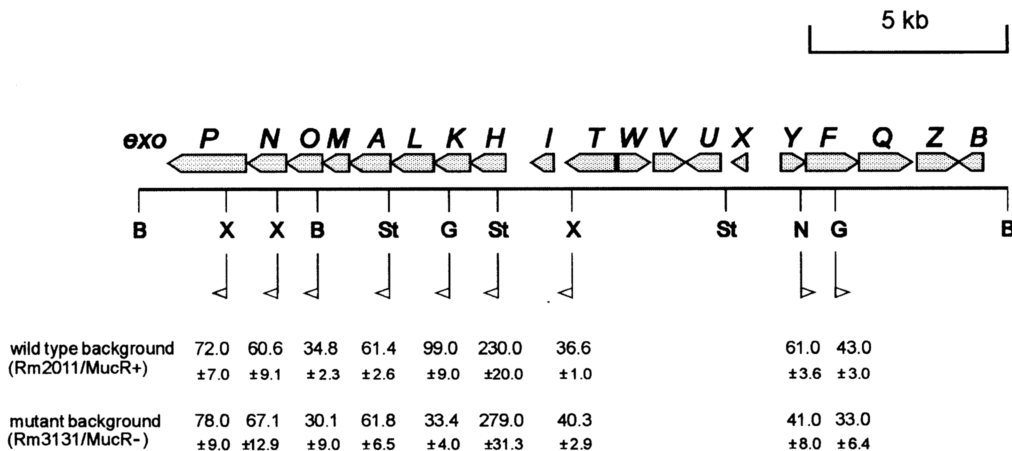

Fig. 7. Expression of *exo* genes in a MucR$^+$ and a MucR$^-$ background. The location of *lacZ* insertions in the *exo* gene region and β-galactosidase activities of *exo-lacZ* transcription fusions in the wild-type (Rm2011) and the *mucR*$^-$ (Rm3131) background are given. The respective *Rhizobium meliloti* strains are listed in Table 1. The flags show the orientation of the *lacZ* gene. Controls without *lacZ*-Gm^r interposon: Rm2011 , 3.9 ± 1.7 and Rm3131, 1.1 ± 0.9. Abbreviations: B, *Bam*HI; G, *Bgl*II, N, *Not*I; St, *Stu*I; X, *Xho*I.

mutants Rm3131 and RmJQ46, we found that 3 weeks after inoculation only Rm3131 induced nitrogen-fixing nodules on alfalfa, the double mutant RmJQ46 did not. We assume that the traces of EPS I produced by mutant Rm3131 can account for nodule infection.

R. meliloti mutants exclusively producing EPS II have also been described by Walker and Glazebrook (1989) and Zhan et al. (1989). Regarding the symbiotic properties, these mutants were at least partially able to form a normal nitrogen-fixing symbiosis. Therefore, the authors concluded that EPS II can replace EPS I in the symbiosis with the host plant. Their results are evidently in contrast to our findings concerning the symbiotic phenotype of the EPS II producing mutant RmJQ46. These discrepancies might be explained by different nodulation assays. Walker and Glazebrook as well as Zhan and coworkers kept the plant roots in the dark and inspected the nodules after 4 weeks. In contrast, the root system in our nodulation assay was exposed to light, which might have negatively influenced nodule formation.

To analyze the molecular basis of the mutation in strain Rm3131 we isolated and sequenced a 3.6-kb *Eco*RI fragment of the *R. meliloti* chromosome complementing mutant Rm3131. We identified six open reading frames. The Tn*5* insertion of mutant Rm3131 could be assigned to ORF 3. The function of the other ORFs remains unknown since no mutant phenotype or homology to any known gene was detected. By complementation and hybridization experiments we found that mutant Rm3131 is affected in the same locus as the *mucR* mutant Rm7010 previously described by Zhan et al. (1989). Hence, ORF 3 was termed *mucR*.

The *mucR* gene encodes a protein with a potential zinc finger motif of the C_2H_2-type. This motif is present in a number of eukaryotic transcription factors like TFIIIA and is proposed to function as a DNA binding domain (Berg 1990). We found a striking homology of MucR to the *A. tumefaciens* Ros protein which also contains this zinc finger motif (Cooley et al. 1991). Ros is positively regulating the succinoglycan synthesis. Additionally, Ros is a negative regulator of the *virC* and *virD* operons (Cooley et al. 1991). Hybridization experiments with a *mucR* gene probe revealed homologous genes also in other rhizobacteria which may indicate a wider distribution of this regulatory gene.

In *R. meliloti*, additional regulators of the succinoglycan biosynthesis have been found. Insertions in *exoS* and *exoR* resulted in an increase in EPS I production (Doherty et al. 1988) and for ExoR it was shown that it acts as a negative regulator of the transcription of the *exo* genes (Reed et al. 1991b). Using *lacZ* fusions we studied the influence of MucR

	Genotype		Strain	*mucR-lacZ* expression
1	E 81 26 E // E E; *mucR**, *mucR*		Rm2011-pAR26	211 ± 4
			Rm2011-pAR81	226 ± 40
2	E 81 26 E; *mucR**		Rm26	1031 ± 61
			Rm81	970 ± 131
3	E 81 26 E; *mucR**	E *mucR* E; pAR360 ~100x	Rm26 (pAR360)	112 ± 26
			Rm81 (pAR360)	117 ± 19
4	E 81 26 E; *mucR**	E *mucR** E; pAR361 ~100x	Rm26 (pAR361)	911 ± 138
			Rm81 (pAR361)	898 ± 99
5	26; E *mucR** E; pAR26 ~50x	Sp *mucR* X; pAR300 ~70x	*E. coli* MC1000 (pAR300, pAR26)	313 ± 19
6	26; E *mucR** E; pAR26 ~50x	—	*E. coli* MC1000 (pAR26)	613 ± 14

Fig. 8. Autoregulation of the *mucR* gene in an *Rhizobium meliloti* and *Escherichia coli* background. A schematic drawing of the constructs is given. Black bars represent functional *mucR* genes, open bars indicate mutated *mucR* genes (only the upstream regions are present). The *mucR-lacZ* expression is given in β-galactosidase units. The copy number of the plasmids is indicated. Controls without *lacZ*-Gm^r interposon: *R. meliloti* Rm2011, 6 ± 2 and *E. coli* MC1000, 5 ± 2. Abbreviation: E, *Eco*RI; Sp, *Sph*I; X, *Xho*I.

on the transcription of the *exo* genes. We found no significant influence of MucR on *exo* gene transcription, with the exception of *exoK*, *exoF*, and *exoY*. The *exoK* transcription was drastically reduced to one third in a MucR$^-$ background, whereas the transcription of *exoF* and *exoY* was only slightly reduced. Since Leigh and Walker (1994) reported that mutations in *mucR* decreased the expression of the *exo* genes we conclude that MucR is regulating the EPS I synthesis post-transcriptionally. One possibility for this type of regulation may be the binding of MucR to mRNA and hence influencing the mRNA stability. Another possibility may be a posttranslational regulation of EPS I biosynthesis as it was discussed for ExoX (Müller et al. 1993; Leigh and Walker 1994).

In contrast to *exo* gene expression, we found a pronounced negative effect of MucR on the transcription of *exp* genes. We therefore assume that MucR may act as a repressor of *exp* gene transcription. The negative effect also explains why the *R. meliloti* wild-type strain Rm2011 does not produce EPS II.

Additionally, our transcription analyses revealed that *mucR* is negatively autoregulated. This was also reported for the homologous *ros* gene of *A. tumefaciens* (Cooley et al. 1991). For the Ros protein it was shown that it binds to a *ros* consensus sequence (*ros* box) located upstream of *ros* and the Ros regulated *virC* and *virD* operons (D'Souza et al. 1993). The *ros* box is organized as an inverted repeat. We identified a sequence homologous to the *ros* box upstream of the *mucR* gene and propose that this DNA sequence may function as a binding site of MucR responsible for the autoregulation of the *mucR* gene. Similar binding sites might be located upstream of the negatively regulated *exp* genes, but DNA sequences of *exp* genes have not been published yet.

The results of this report showed that MucR is an important component of the regulatory network of EPS synthesis in *R. meliloti*. Further analysis of MucR and its interaction with nucleic acids will reveal the mode of its regulatory properties.

MATERIALS AND METHODS

Bacterial strains and plasmids.

E. coli and *R. meliloti* strains, plasmids and phages used in this study are listed in Tables 2 and 3. *Rhizobium* sp. NGR234

Table 2. Strains used and constructed for this work

Strain	Relevant characteristics	Source or reference
E. coli		
DH5α	*recA*1, Δ*lac*U169, 80δ*lacZ*ΔM15	Bethesda Research Laboratories
S17-1	MM294, RP4-2-Tc::Mu-Km::Tn*7* chromosomally integrated	Simon et al. 1983
MC1000	Δ[*lacIZY*], *strA*	Casadaban and Cohen 1980
JQ101R	HB101, Rif^r	Müller et al. 1988
R. meliloti		
Rm2011	Wild-type, Nod$^+$ Fix$^+$ Inf$^+$ EPS$^+$ Cfw$^+$ Sm^r	Casse et al. 1979
Rm3131	Rm2011, *mucR*31::Tn*5*	This work
RmJQ13	Rm2011, *exoY*::Tn*5*-Gm	Kapp et al. 1990
RmJQ46	Rm2011, *exoY*::Tn*5*-Gm, *mucR*31::Tn*5*	This work
Rm2011-pAR26	Rm2011 with integrated pAR26	This work
Rm2011-pAR81	Rm2011 with integrated pAR81	This work
Rm26	Rm2011, *mucR*26::Tn*5*-B20	This work
Rm81	Rm2011, *mucR*81::Tn*5*-B20	This work
Rm26-pAR360	Rm26 with integrated pAR360	This work
Rm81-pAR360	Rm81 with integrated pAR360	This work
Rm26-pAR361	Rm26 with integrated pAR361	This work
Rm81-pAR361	Rm81 with integrated pAR361	This work
Rm7010	*mucR*12::Tn*5*-233, Gm^r, Sp^r	Zhan et al. 1989
Rm8605	*expA*125::Tn*5*, *thi*-502::Tn*5*-11, Nm^r	Glazebrook and Walker 1989
RmAR9000	Rm2011, *expA*125::Tn*5*	This work
RmAR1011	Rm2011, *expA*11-*lacZ*-Gm^r	This work
RmAR1012	Rm2011, *expA*12-*lacZ*-Gm^r	This work
RmAR1013	Rm2011, *expA*13-*lacZ*-Gm^r	This work
RmAR1111	Rm3131, *expA*11-*lacZ*-Gm^r	This work
RmAR1112	Rm3131, *expA*12-*lacZ*-Gm^r	This work
RmAR1113	Rm3131, *expA*13-*lacZ*-Gm^r	This work
RmH1a	Rm2011, *exoH*-*lacZ*-Gm^r	Becker et al. 1993b
RmH2a	Rm2011, *exoK*-*lacZ*-Gm^r	Becker et al. 1993b
RmH11a	Rm2011, *exoT*-*lacZ*-Gm^r	Becker et al. 1993c
RmH4a	Rm2011, *exoA*-*lacZ*-Gm^r	Becker et al. 1993b
RmH6a	Rm2011, *exoN*-*lacZ*-Gm^r	Becker et al. 1993b
RmH5a	Rm2011, *exoO*-*lacZ*-Gm^r	Becker et al. 1993b
RmH7a	Rm2011, *exoP*-*lacZ*-Gm^r	Becker et al. 1993b
RmAR9007	Rm2011, *exoY*-*lacZ*-Gm^r	This work
RmAR9008	Rm2011, *exoF*-*lacZ*-Gm^r	This work
RmAR9010	Rm3131, *exoH*-*lacZ*-Gm^r	This work
RmAR9011	Rm3131, *exoK*-*lacZ*-Gm^r	This work
RmAR9012	Rm3131, *exoT*-*lacZ*-Gm^r	This work
RmAR9013	Rm3131, *exoA*-*lacZ*-Gm^r	This work
RmAR9014	Rm3131, *exoN*-*lacZ*-Gm^r	This work
RmAR9015	Rm3131, *exoO*-*lacZ*-Gm^r	This work
RmAR9016	Rm3131, *exoP*-*lacZ*-Gm^r	This work
RmAR9017	Rm3131, *exoY*-*lacZ*-Gm^r	This work
RmAR9018	Rm3131, *exoF*-*lacZ*-Gm^r	This work

was obtained from W. J. Broughton (Université de Genève, Switzerland), *R. fredii* USDA205, *R. l.* bv. *trifolii* RS24, and *R. l.* bv. *phaseoli* CFN42 were from A. M. Buendia (Facultad de Biologia, Sevilla, Spain). *Erwinia stewartii* SW2 and *Pseudomonas fluorescens* were obtained from R. Eichenlaub (Universität Bielefeld, Germany). *R. l.* bv. *viciae* VF39 and *Agrobacterium tumefaciens* LBA4404 are from the strain collection of the Lehrstuhl für Genetik, Universität Bielefeld.

Media and growth conditions.

E. coli strains were grown in Penassay broth (Difco Laboratories, Detroit, Michigan) or in LB medium (Maniatis et al. 1982) at 37°C. *R. meliloti* strains were grown on TY medium (Beringer 1974) with 0.4 g of $CaCl_2$ per liter, M98 medium (Keller et al. 1988) or LB medium (Maniatis et al. 1982) supplemented with 2.5 mM $CaCl_2$ and 2.5 mM $MgSO_4$ at 30°C.

Antibiotics were supplemented as required at the following concentrations (mg/liter): for *R. meliloti*: streptomycin, 600; nalidixic acid, 8; tetracycline, 7; gentamicin, 40; and neomycin, 120; for *E. coli*: tetracycline, 7; ampicillin, 120; gentamicin, 8; and kanamycin, 50.

DNA biochemistry.

Plasmid DNA from *E. coli* was prepared as described by Priefer (1984). DNA restriction, cloning procedures and agarose gel electrophoresis followed established protocols (Maniatis et al. 1982). Transformation of *E. coli* cells was performed according to Morrison (1977). Total DNA from *R. meliloti* and other bacteria was isolated according to Meade et al. (1982).

Southern hybridization.

Total DNA was restricted, electrophoretically separated in a 0.8% Tris-acetate agarose gel, and vacuum-blotted, according to the supplier's instructions (Pharmacia, Freiburg, Germany) onto a nylon filter (Hybond N^+; Amersham Buchler GmbH, Braunschweig, Germany). The DNA hybridization of digoxigenin labeled probes (Boehringer-Mannheim, Germany) was carried out according to the manufacturer's instructions for high stringency conditions (68°C, 5× SSC). For low stringency conditions hybridization was performed at 60°C in 7× SSC (1× SSC = 0.15 M NaCl, 0.015 M Na-citrate, pH 7.0).

DNA sequencing.

The 3.6-kb *Eco*RI insert of plasmid pMS136 was cloned into the sequencing vector pSVB30 (Arnold and Pühler 1988) in both orientations. Appropriate subclones for sequencing were constructed by creating a set of nested deletions (Henikoff 1984). The DNA sequence was obtained for both strands by the chain termination method (Sanger et al. 1977) using double-stranded DNA (Arnold et al. 1988). The Tn*5*-B20 insertion junctions were sequenced using an oligonucleotide primer complementary to the first nucleotides of the *lacZ* gene integrated in IS*50*L (Keller et al. 1988; Simon et al. 1989). The Tn*5* insertion junction of mutant Rm3131 was sequenced using an oligonucleotide primer complementary to the first nucleotides of IS*50*L.

Table 3. Plasmids and phages used and constructed in this work

Plasmid/phage	Relevant characteristics	Source or reference
pSVB30	pUC8 derivative, Ap^r	Arnold and Pühler 1988
RP4-Gm	RP4 derivative, Km^S, Tc^S, Gm^r	Klipp et al. 1988
pK18	pUC18 derivative, Km^r	Pridmore 1987
pK18*mob*	pUC18 derivative, *mob*, Km^r	Schäfer et al. 1994
pHP45 *X*	pBR322 derivative, Sp^r	Fellay et al. 1987
pSUP102	pACYC184 derivative, *mob*, Tc^r, Cm^r	Simon et al. 1985
pSUP102-18	pSUP102 derivative carrying the mcs/*lacZa Pvu*II fragment of pK18; *mob*, Tc^r, Cm^S	This work
pSUP104	pACYC184 derivative, *mob*, *oriV* (RSF1010), Tc^r, Cm^r	Priefer et al. 1985
pSUP205	pBR325 derivative, *mob*, λ *cos* site, Cm^r, Tc^r	Simon et al. 1983
cJQ1	pSUP205 cosmid with chromosomal *Eco*RI fragments of Rm2011 complementing Rm3131 (Fig. 3)	This work
cJQ2	pSUP205 cosmid with chromosomal *Eco*RI fragments of Rm2011 complementing Rm3131 (Fig. 3)	This work
pMS136	pSUP102 with 3.6 kb *Eco*RI *mucR* fragment	This work
pAR26	pMS136, *mucR*26::Tn*5*-B20	This work
pAR81	pMS136, *mucR*81::Tn*5*-B20	This work
pAR300	pHP45 X with 1.2 kb *Sph*I-*Xho*I *mucR* subfragment	This work
pAR360	pSUP104 with the 3.6 kb *Eco*RI *mucR* fragment	This work
pAR361	pAR360 derivative, the *Tth*111I site in *mucR* filled in	This work
pAB2001	pUC6S derivative with a *lacZ*-Gm^r interposon	Becker et al. 1993b
pAR50	pSUP102-18 with a 14.7 kb *Kpn*I *expA*125::Tn*5* fragment	This work
pAR100	pSUP102-18 with a 8.9 kb *Kpn*I *expA* fragment	This work
pAR1	pK18*mob* with a 8.9 kb *Kpn*I *expA* fragment	This work
pAR1-2	pK18*mob* with a 3 kb *Bgl*II-*Kpn*I *expA* subfragment	This work
pAR111	pAR1-2 derivative, *expA*-*lacZ*-Gm^r; insertion site: *Hin*dIII	This work
pAR112	pAR1-2 derivative, *expA*-*lacZ*-Gm^r; insertion site: *Hin*dIII *lacZ*-Gm^r interposon in opposite direction as in pAR111	This work
pAR113	pAR1 derivative, *expA*-*lacZ*-Gm^r; insertion site: *Bgl*II	This work
pRmPM157.8	pSUP102 with a 7.8 kb *Eco*RI fragment carrying *exo* genes	Müller et al. 1988
pAR578	pK18*mob* with the 7.8 kb *Eco*I fragment of pRmPM157.8	This work
pAR447	pK18*mob* with a 4.47 kb *Cla*I *exoYF* fragment of pRmPM157.8	This work
pAR907	pAR447 derivative, *exoY*-*lacZ*-Gm^r; insertion site: *Not*I	This work
pAR908	pAR578 derivative, *exoF*-*lacZ*-Gm^r; insertion site: *Bgl*II	This work
λ::Tn*5*-B20	λ-Derivative carrying the Tn*5*-*lacZ* transposon Tn*5*-B20	Simon et al. 1989
ΦM12	*R. meliloti* transducing phage	Finan et al. 1984

Isolation of the *R. meliloti expA* complementation group.

The *expA*125::Tn*5* region of *R. meliloti* mutant Rm8605 (Glazebrook and Walker 1989) was transduced via ΦM12 (Finan et al. 1984) into the *R. meliloti* wild-type strain Rm2011 resulting in strain RmAR9000. From strain RmAR9000 *Kpn*I restricted total DNA was cloned into vector pSUP102-18 and screened for a Tn*5* (Km^r) carrying insert. One plasmid, pAR50, carried a 14.7-kb *Kpn*I DNA fragment with the *expA*125::Tn*5* insertion.

The wild-type *expA Kpn*I fragment was isolated by in vivo cloning. Plasmid pAR50 was transferred to *R. meliloti* Rm2011 using the filtercrossing method (Simon 1984) and integrated into the *expA* region, resulting in strain Rm2011-pAR50. Subsequently, RP4-Gm was transferred to Rm2011-pAR50 to mobilize, after desintegration of the vector together with the native *expA* region, the resulting plasmid pAR100 to *E. coli* JQ101R. Following several purification steps, plasmid pAR100 carrying the native 8.9-kb *Kpn*I *expA* fragment was verified by restriction analysis. For further experiments, the 8.9-kp *Kpn*I fragment and a 3-kb *Bgl*II–*Kpn*I subfragment were cloned into plasmid pK18*mob*, resulting in plasmids pAR1 and pAR1-2, respectively.

Construction of *lacZ* fusions applying transposon and interposon mutagenesis

Tn*5*-B20 (Tn*5*-*lacZ*) mutagenesis of plasmid pMS136 was carried out in *E. coli* as described by Keller *et al.* (1988) and Simon et al. (1989). For the construction of *lacZ* fusions with *exoY*, *exoF*, and *expA*, the promoterless *lacZ*-Gm^r interposon of plasmid pAB2001 (Becker et al. 1993b) was used. The interposon was inserted into *exoF* in the direction of transcription using the single *Bgl*II site of plasmid pAR578 which resulted in plasmid pAR908. An *exoY–lacZ* fusion was achieved by cloning the interposon in the direction of transcription into the single *Not*I site of plasmid pAR447 resulting in plasmid pAR907. To obtain *expA–lacZ* fusions, the interposon was integrated in the direction of transcription into the *Bgl*II site of plasmid pAR1 (giving plasmid pAR113), and in both orientations into the *Hin*dIII site of plasmid pAR1-2, resulting in plasmids pAR111 and pAR112. All transposon and interposon insertions were established in *R. meliloti* Rm2011 via homogenotization as described in Keller *et al.* (1988). Additionally, the *expA–lacZ* fusions of plasmids pAR111, pAR112, and pAR113 were established in the same way in mutant Rm3131. *R. meliloti* strains RmAR9010 to RmAR9018 (Table 1) were obtained via ΦM12 transduction (Finan et al. 1984) of the *mucR*::Tn*5* DNA region of mutant Rm3131 to the *exo–lacZ*-Gm^r fusion strains RmH1a, RmH2a, RmH11a, RmH4a, RmH6a, RmH5a, RmH7a, RmAR9007, and RmAR9008, respectively. The resulting strains were verified by Southern hybridization.

Exopolysaccharide production, NMR spectroscopy, and plant nodulation assay.

EPS production and symbiotic properties of *R. meliloti* strains were assayed as described by Müller et al. (1993). The NMR spectroscopy was performed as described by Müller et al. (1988) with the exception that 90 mg of freeze-dried EPS II were dissolved in 7 ml of D_2O. The ^{13}C spectra were taken at 80°C.

β-Galactosidase assay.

R. meliloti strains carrying Tn*5*-B20 or *lacZ*-Gm^r insertions were assayed for β-galactosidase activity as previously described by Becker et al. (1993c). *E. coli* strains carrying plasmids with *lacZ*-Gm^r insertions were analyzed according to Miller (1972).

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