Genetic Analysis of the *rkp-3* Gene Region in *Sinorhizobium meliloti* 41: *rkpY* Directs Capsular Polysaccharide Synthesis to K_R5 Antigen Production

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Rhizobial surface polysaccharides, including capsular polysaccharides (KPS), are involved in symbiotic infection. The *rkp-3* locus of *Sinorhizobium meliloti* 41 is responsible for the production of pseudaminic acid, one of the components of the K_R5 antigen, a strain-specific KPS. We have extended the sequence determination and genetic dissection of the *rkp-3* region to clarify the structure and function of the *rkpY* gene and to identify additional *rkp* genes. Except for *rkpY*, no other genes were found where mutation affected the KPS structure and symbiosis. These mutants show a unique phenotype producing a low molecular weight polysaccharide (LMW PS). Creating double mutants, we have shown that biosynthesis genes of the K_R5 antigen except *rkpZ* are not necessary for the production of this LMW PS. Polysaccharide analysis of genetically modified strains suggests that *rkpY* has pleiotropic effects on polysaccharide production. It directs KPS synthesis to the K_R5 antigen and influences lipo-oligo 3-deoxy-d-manno-2 octulosonic acid (Kdo) production in *S. meliloti* 41. In addition, *rkpY* suppresses the lipo-oligoKdo production when it is introduced into *S. meliloti* 1021.

Gram-negative soil bacteria of *Rhizobiaceae* family belong to the α -proteobacteria and contain the N₂-fixing endosymbionts of legumes (*Rhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Sinorhizobium*, and *Mezorhizobium* spp.) called rhizobia. Symbiotic plant–bacterium interaction begins with the induction of bacterial nodulation (*nod*) genes by specific plant flavonoids and with the initiation of a new plant organ, the nodule, in a host-specific manner by the product of Nod proteins. Invasion of the developing nodule by the bacteria is also a host-specific event where several additional bacterial genes are needed. Cell-surface polysaccharides of rhizobia were shown to be involved in several steps of the nodulation process, such as infection thread initiation and development, nodule invasion, and host specificity. However, unlike Nod factors, their

specific functions have not been elucidated (Frayssé et al. 2003; Jones et al. 2007).

Sinorhizobium meliloti produces several types of surface polysaccharides, including exopolysaccharides (EPS I and EPS II), lipopolysaccharide (LPS), cyclic glucans, and capsular polysaccharides (KPS or K-antigen). All of these components may be involved in the invasion process because mutant bacteria unable to produce one of these polysaccharides and to propagate within the infection thread were reported by several laboratories (Frayssé et al. 2003; Jones et al. 2007). *S. meliloti* 41 produces a strain-specific KPS, the K_R5 antigen, which functionally compensates for the EPS I (succinoglycan) during the infection process, as was shown by using an EPS-deficient *exoB* mutant, AK631 (Putnoky et al. 1990; Petrovics et al. 1993). In an EPS-defective genetic background, the K_R5 antigen of *S. meliloti* 41 is essential for the normal development of the infection threads and for the invasion of host plants. Mutants lacking the K_R5 antigen produced aborted infection threads and failed to develop nitrogen-fixing symbiosis. Recently, the structure and genetics of this antigen were mainly elucidated. It is composed of low and high molecular weight (LMW and HMW) polysaccharides containing glucuronic acid (GlcA) and pseudaminic acid (Pse) disaccharide subunits which are decorated by butyryl and acetyl residues. This structure is analogous to the group II K antigens from *Escherichia coli* (Reuhs et al. 1993, 1998). A similar KPS structure was reported recently in *Rhizobium* sp. strain NGR234 (Le Quere et al. 2006) that also influences the development of symbiosis.

In *S. meliloti* 41, three *rkp* gene clusters (*rkp-1*, *rkp-2*, and *rkp-3*) involved in the K_R5 antigen biosynthesis were identified (Putnoky et al. 1990; Kereszt et al. 1998; Kiss et al. 2001). Almost all of the *rkp* mutants show the same phenotypes. They are not able to i) produce wild-type KPS as the biochemical and immunological analysis indicated, ii) invade host plants in AK631 background, and iii) adsorb strain-specific bacteriophage 16-3 (Petrovics et al. 1993; Kereszt et al. 1998; Kiss et al. 1998).

The *rkp-1* region harbors genes (*rkpA* to *rkpF*) responsible for the synthesis of a specific lipid carrier for KPS biosynthesis, and genes (*rkpG* to *rkpJ*) involved in the modification and transfer of the carrier's lipophilic molecules (Petrovics et al. 1993; Kiss et al. 1997). Among these genes, *rkpF* encoding an acyl carrier protein was studied more in detail (Epple et al. 1998). Interestingly, the *rkpA* to *rkpF* genes have been fused

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into a single large open reading frame (ORF) in strain *S. meliloti* 1021 (accession number AL591784) and in *S. fredii* HH103. In these strains, an additional *rkp* gene encoding a putative export protein (*rkpU*) was identified upstream of *rkpA* (Parada et al. 2006). The *rkpU* sequence of *S. meliloti* 41 was also determined (accession number AF247710) but its exact position remained unknown.

The *rkp-2* region carries two genes (*lpsL* and *rkpK*), both necessary for wild-type LPS production. Moreover, *rkpK* was shown to be required for the synthesis of a K_{R5} antigen precursor (UDP-GlcA) by encoding a UDP-glucose dehydrogenase (Kereszt et al. 1998).

In contrast to the above chromosomal regions, the *rkp-3* region lies on the pRme41c megaplasmid (pSymB) and contains several polysaccharide biosynthesis genes localized by genetic and DNA sequence analysis (Kereszt et al. 1998; Kiss et al. 2001). The *rkpLMNOPQ* gene cluster presumably performs the synthesis of the Pse component of the K_{R5} antigen. Three additional genes (*rkpR*, *rkpS*, and *rkpT*) encode for putative products with a high degree of similarity to polysaccharide transport proteins, but have no effect on either KPS biosynthesis or symbiosis. The right part of the published sequence (Kiss et al. 2001) encodes for RkpZ, which is homologous to polysaccharide chain-length-determinant proteins. The *rkpZ* insertional mutants show a size range modification of KPS (Reuhs et al. 1995). Outside of the sequenced region, approximately 6 kb downstream from *rkpZ*, an additional gene, *rkpY*, was identified by Tn5 insertions. These mutations affected the K_{R5} antigen production and resulted in invasion-defective and phage-16-3-resistant bacteria. A unique polysaccharide pattern was characteristic for these mutant strains (AT232 and AT233) (Kereszt et al. 1998). They produced only an LMW Alcian blue-staining material, supposed to represent an LMW derivative of the K_{R5} antigen.

In this study, we completed the sequence determination and genetic analysis of the *rkp-3* region and showed by genetic experiments and polysaccharide analysis that *rkpY* mutants

probably synthesize oligo 3-deoxy-d-manno-2 octulosonic acid (Kdo) but fail to produce K_{R5} antigen.

RESULTS

Nucleotide sequence of the *rkpY* region.

In order to clarify the structure of the *rkpY* gene and the existence of additional coding frames, we have determined the DNA sequence of a 6-kb *Bam*HI fragment (AJ249130). One large ORF encoding a 1,024-amino-acid (aa)-long putative protein (116 kDa) was found in this region. All three Tn5 insertions (Kereszt et al. 1998; Kiss et al. 2001) that destroyed K_{R5} antigen production in this region were localized within this ORF by cloning and sequence analysis. Our results proved that this ORF represents the *rkpY* gene (Fig. 1A and B). Computer analysis of the putative RkpY protein highlighted no similarity to other protein sequences, and no characterized motifs or other functional domains were detected. The only structural characteristics of RkpY were one or two putative transmembrane domains. These data suggest that the *rkpY* gene encodes for a novel type of protein.

Using additional subclones (data not shown), we have determined 12,289 nucleotide sequences in total (AM849044) expanding the DNA sequence of the *rkp-3* region published earlier (Kiss et al. 2001). An ORF map of the extended *rkp-3* region is shown in Figure 1A. In the newly sequenced part, eight ORF were found. In addition to *rkpY*, a homologue of *rkpT* (*rkpT2*) represents a possible polysaccharide biosynthesis gene. The predicted product of this gene is an ABC-2 transporter permease, possibly involved in a cell surface polysaccharide export. It shares the highest identity (58%) with RkpT1 of *S. meliloti* 1021. However, a comparison of the related protein sequences showed that the putative RkpT2 protein of *S. meliloti* 41 is more related to RkpT1 sequences from *S. meliloti* 1021, *S. medicae* WSM419, and *Rhizobium* sp. strain NGR234 than to the RkpT2 proteins from these species. The transmembrane prediction of RkpT2 showed five

Fig. 1. Genetic map of the extended *rkp-3* region in *Sinorhizobium meliloti* 41. **A**, Open reading frames (ORF) in the *rkp-3* region. Black arrows represent genes involved in the biosynthesis of K-antigen; gray arrows show ORF encoding hypothetical proteins with unknown function; white arrows represent ORF encoding proteins with homology to those encoded in insertion elements ISRm26 and ISRm15. **B**, Positions of the natural and generated insertion sequences in the region. A complete copy of ISRm26 disrupts ORF ISRm15-A. Full-length ORF ISRm15-B and a truncated copy of ISRm15-C sequences lay downstream from the ISRm26 insertion. Vertical bars with knobs represent the positions of the generated Tn5(66), Tn5(232), and Tn5(35) insertions. Simple vertical bars represent entranceposon insertions isolated in this study. Numbers show the exact coordinates of the insertions according to the unified *rkp-3* sequence. **C**, Full lines represent inserts of different cosmid or plasmid derivatives used in this work. pAT399 and pAT401 are cosmid derivatives (Kiss et al. 2001); pBluescriptII SK(+) derivatives: *Nco*I fragment (pAV433), *Bam*HI fragment (pPP2543), *Eco*RI fragments (pPP4166, pPP4170); pBBR1-MCS2 derivatives: *Bam*HI fragment (pNE3265), *Sal*I fragments (pAV568, pAV571), pBBR1-MCS5 derivative: *Bam*HI-*Pst*I fragment (pAV579). **D**, Range of sequences in the database records under the given accession numbers. The two records have 86 bp of overlap.

to seven possible domains similar to that of RkpT1 of *S. meliloti* 41.

The putative product of *orf7343* (Fig. 1A) possesses a methyltransferase_11 domain (Pfam entry PF08241) and shares 72% identity with the predicted product of *rkp3_012* from *Rhizobium* sp. strain NGR234 (ABD15245). The product of another putative gene, *orf8077*, harbors an ATP-binding region signature of protein kinases (ProSite entry PS00107) but has no homologues in the databases.

One complete insertion element (ISRM26) and a degraded copy of ISRM15 were identified upstream of the *rkpY* gene (Fig. 1B). ORF ISRM26-1, ISRM26-2, and ISRM26-3 and the 7-bp inverted repeat ends represent a complete copy of ISRM26. The 9-bp direct repeats of the target sequence were also identified. These findings suggest that this copy of ISRM26 is a functional one. Interestingly, it has been inserted into a truncated copy of ISRM15 sequence containing only one complete coding region, ISRM15-B (*protB*). The ISRM15-A (*protA*) sequence was disrupted by the ISRM26, whereas the larger part of ISRM15-C (*protC*) sequence at the 3' end probably was removed earlier by a deletion event (Fig. 1B).

We performed an *in silico* search for the homologues of the *rkp-3* genes. Several gene clusters similar to *rkpLMNOPQ* were identified in different representatives of proteobacteria (data not shown). The putative product of these genes show homology to that of the *pseB*, *pseC*, *pseF*, *pseG*, *pseH*, and *pseI* genes of *Campylobacter jejuni* and *Helicobacter pylori*, where the biochemistry and genetics of Pse biosynthesis were studied in detail (Schirm et al. 2003; Guerry et al. 2006; Schoenhofen et al. 2006). Interestingly, as mentioned above, no homologues of *rkpY* were found either in the vicinity of the putative *pse* gene clusters or anywhere in the complete genome sequences available in nucleotide databases. The only exception is *Rhizobium* sp. strain NGR234, where the presence of *rkpY* close to other *rkp* genes were reported recently (Le Quere et al. 2006).

Mutational analysis of the *rkpY* region.

To investigate the possible function of the newly identified ORF (*orf7343*, *orf8077*, and *rkpT2*) in polysaccharide biosynthesis and symbiosis, a Mu entranceposon insertion mutagenesis was applied on appropriate subclones (Fig. 1C). Insertions car-

rying kanamycin resistance genes were physically mapped and sequenced. Representative insertions in the above ORF (Fig. 1B) were chosen to create homologous recombinants (as described below). Mutant bacteria were tested for their symbiotic ability, phage 16-3 resistance, and KPS or LPS production. No insertion resulting in any mutant phenotype was found, suggesting that *orf7343*, *orf8077*, and *rkpT2* have no direct function in either polysaccharide production or symbiosis or the propagation of phage 16-3. Another reason for the lack of mutant phenotype could be functional redundancy elsewhere in the genome (Fig. 1A, *rkpT1* and *rkpT2*).

In order to show whether the putative membrane proteins in the *rkp-3* region harbor functional transmembrane domains, we performed *phoA* fusion experiments. Alkaline phosphatase (PhoA) is activated in the periplasmic space. The promoterless *phoA* gene of Tn*phoA* transposon lacks its 5' end coding for the signal peptide; therefore, most insertions result in no enzyme production. Translational fusion will only generate alkaline phosphatase activity if the protein fused to the truncated PhoA peptide contains one or more transmembrane domains that orient the C-terminal alkaline phosphatase part to the periplasm. Transposon Tn*phoA* insertions were generated on cosmid pAT401 (Fig. 1C), and the target sequences of the insertions resulting in alkaline phosphatase activity in *S. meliloti* 41 were determined. Evaluating the sequence data, we could localize active translational fusions in the RkpM protein (after amino acid positions 70 and 105) (Fig. 2), the RkpR protein (after amino acid positions 46, 140, and 186) (Fig. 2), and the RkpT2 protein (after amino acid position 90).

However, all of our efforts to isolate active RkpY-PhoA fusions failed, although the *rkpY* gene is three times longer than any of the above coding regions. The reason for this negative result may be that RkpY has either no transmembrane domains at all or a single C-terminal domain that is not suitable to isolate PhoA fusions by the method applied (Fig. 2) (discussed below).

RkpY plays a direct role in phage 16-3 infection.

Recently, we have described a special *rkpM* missense mutation that blocked the infection of the wild-type 16-3 phage but which made the isolation of host range phage mutants possible (Putnoky et al. 2004). We could also identify one spontaneous mutation in the *rkpY* gene that resulted in a similar phenotype (PP4073; discussed below). In order to delimit the position of the mutation, marker rescue experiments were carried out. We took advantage of our observation that phage M1 of *S. meliloti* 1021 could infect the *rkp* mutants of *S. meliloti* 41 (Putnoky et al. 1990). Therefore, we examined which subfragment of the *rkpY* gene generates phage M1-resistant wild-type bacteria by homologous recombination. Of the three subclones (Fig. 1C; pAV568, pAV571, and pAV579), only pAV571, representing the middle part of the gene, increased the appearance of M1-resistant recombinants, suggesting that this fragment covers the mutation. After amplifying this region from strain PP4073, one missense mutation, a CCG codon instead of CTG, was identified that resulted in the L552P substitution (Fig. 2). The fact that a missense mutation of *rkpY* ensures the isolation of host range phage mutants supports the idea that the RkpY protein is involved directly in bacterium-phage recognition.

Second mutations in *rkp* genes except *rkpZ* do not affect the LMW polysaccharide production in *rkpY* mutants.

To test whether the LMW polysaccharide (LMW PS) produced by the *rkpY* mutants is a derivative of the K_R5 antigen, as we previously supposed, we mutated essential *rkp* genes in an *rkpY* mutant background. For this purpose, an *rkpY* spontaneous mutant, NE3222, was identified (as described below).

Fig. 2. Suggested structures of predicted transmembrane proteins RkpM, RkpR, and RkpY. Numbers represent i) the coordinates of the putative transmembrane domains (gray boxes) in amino acid residues, ii) the insertion of the PhoA fusions (at the arrows), iii) the receptor mutations (at the circles), and iv) the length of the predicted proteins (at the C-terminal ends).

To introduce the second mutations, different clones carrying transposon Tn5 insertions in representative *rkp* genes were applied (Table 1). Homologous recombinants were examined for KPS production by deoxycholate polyacrylamide gel electrophoresis (DOC-PAGE).

Interestingly, the second mutations, except in the *rkpZ* gene, did not influence the LMW PS production of the *rkpY* mutant (Table 1; Fig. 3), suggesting that the *rkp* genes which are otherwise essential for K_{R5} antigen production are not involved in the biosynthesis of the LMW PS. In contrast, the *rkpZ/rkpY* mutant strain (AV476) produced no detectable amount of LMW PS (Fig. 3F). Another double mutant (AV518) was also created using a spontaneous *rkpZ* mutant (PP4180) and an entranceposon mutation of the *rkpY* gene. Again, the DOC-PAGE analysis showed no detectable LMW PS (data not

shown), suggesting that the *rkpZ* gene influences the synthesis of this unknown polysaccharide.

***rkpY* suppresses Kdo homopolymer production in *S. meliloti* 1021.**

In the genome sequence of *S. meliloti* 1021, we could not detect any *rkpY*-related gene. To test how the presence of *rkpY* influences the polysaccharide production of this strain, plasmid pNE3265 was conjugated into the wild-type background. This plasmid carries the *rkpY* gene (Fig. 1C) and it was shown to complement *rkpY* mutations in *S. meliloti* 41 (discussed below). DOC-PAGE analysis (Fig. 3) shows that the polysaccharide pattern of *S. meliloti* 1021 (*rkpY*⁺) differs from that of the wild type. Although the LMW PS in strain 1021 representing lipo-oligoKdo is produced in an abundant amount (Fig. 3G), the

Table 1. Deoxycholate polyacrylamide gel electrophoresis (DOC-PAGE) phenotypes of the *rkp-3* and *rkpY-rkp* double mutants

Strain	Genotype	KPS ^a
AK631	Background strain producing K _{R5} antigen (Petrovics et al. 1993)	wt
PP4073	<i>rkpY</i> ₄₀₇₃ spontaneous mutant of AK631 (<i>rkpY</i> L552P)	<i>rkpY</i>
NE3222	<i>rkpY</i> ₃₂₂₂ spontaneous mutant of AK631	<i>rkpY</i>
PP4180	<i>rkpZ</i> ₄₁₈₀ spontaneous mutant (Putnoky et al. 2004)	<i>rkpZ</i>
AV488	AK631 <i>rkpR</i> ::ET-KanR-3 (7591)	wt
AV486	AK631 <i>rkpS</i> ::ET-KanR-3 (9091)	wt
AV461	AK631 <i>rkpT</i> ::ET-KanR-3 (9821)	wt
AV475	AK631 <i>rkpZ</i> ::ET-KanR-3 (11222)	<i>rkpZ</i>
AV458	AK631 <i>rkpY</i> ::ET-KanR-3 (18387)	<i>rkpY</i>
AV493	AK631 <i>orf7343</i> ::ET-KanR-3 (20447)	wt
AV457	AK631 <i>orf8077</i> ::ET-KanR-3 (21174)	wt
AV490	AK631 <i>rkpT2</i> ::ET-KanR-3 (23345)	wt
PP4141	<i>rkpY</i> ₃₂₂₂ + <i>rkpA</i> ::Tn5(666)	<i>rkpY</i>
PP4140	<i>rkpY</i> ₃₂₂₂ + <i>rkpB</i> ::Tn5(665)	<i>rkpY</i>
PP4139	<i>rkpY</i> ₃₂₂₂ + <i>rkpC</i> ::Tn5(644)	<i>rkpY</i>
PP4199	<i>rkpY</i> ₃₂₂₂ + <i>rkpK</i> ::Tn5(207)	<i>rkpY</i>
PP4201	<i>rkpY</i> ₃₂₂₂ + <i>rkpL</i> ::Tn5(187)	<i>rkpY</i>
PP4202	<i>rkpY</i> ₃₂₂₂ + <i>rkpM</i> ::Tn5(212)	<i>rkpY</i>
PP4200	<i>rkpY</i> ₃₂₂₂ + <i>rkpQ</i> ::Tn5(124)	<i>rkpY</i>
PP3300	<i>rkpY</i> ₃₂₂₂ + pBBR1-MCS2:: <i>rkpY</i> ⁺	wt
AV463	<i>rkpY</i> ₃₂₂₂ + <i>rkpR</i> ::ET-KanR-3(7591)	<i>rkpY</i>
AV477	<i>rkpY</i> ₃₂₂₂ + <i>rkpS</i> ::ET-KanR-3(9091)	<i>rkpY</i>
AV479	<i>rkpY</i> ₃₂₂₂ + <i>rkpT</i> ::ET-KanR-3(9821)	<i>rkpY</i>
AV476	<i>rkpY</i> ₃₂₂₂ + <i>rkpZ</i> ::ET-KanR-3(11222)	no KPS
AV494	<i>rkpY</i> ₃₂₂₂ + <i>orf7343</i> ::ET-KanR-3(20447)	<i>rkpY</i>
AV483	<i>rkpY</i> ₃₂₂₂ + <i>orf8077</i> ::ET-KanR-3(21174)	<i>rkpY</i>
AV491	<i>rkpY</i> ₃₂₂₂ + <i>rkpT2</i> ::ET-KanR-3(23345)	<i>rkpY</i>
AV518	<i>rkpZ</i> ₄₁₈₀ + <i>rkpY</i> ::ET-KanR-3(18387)	No KPS

^a KPS = capsular polysaccharides; wt, wild-type KPS pattern on DOC-PAGE; *rkpZ*, KPS production characteristic for *rkpZ* mutants; *rkpY*, low molecular weight (LMW) KPS production characteristic for *rkpY* mutants; no KPS, neither wild-type KPS nor LMW KPS characteristic for *rkpY* mutants were produced.

Fig. 3. Capsular polysaccharide (KPS) production by the *rkp-3* mutants. **A**, Wild-type KPS from *Sinorhizobium meliloti* 41 (AK631); **B**, *rkpZ* phenotype characteristic for PP4180; **C**, *rkpY* phenotype, prototype *rkpY*::Tn5(232); **D** and **E**, *rkpY* phenotype isolated from double mutants *rkpY-rkpA* (PP4141) and *rkpY-rkpL* (PP4201), respectively; **F**, KPS pattern of the *rkpY-rkpZ* double mutant AV476; **G**, *S. meliloti* 1021 and **H**, *S. meliloti* 1021 carrying the *rkpY*⁺ gene on pNE3265; arrow, predicted position of Kdo homopolymer (Sharypova et al. 2006). LMW and HMW = low and high molecular weight.

introduction of *rkpY* resulted in a 10-fold reduction of this production (Fig. 3H). In the LPS pattern, no changes were detected (data not shown). Thus, the *rkpY* gene from *S. meliloti* 41 is able to suppress the lipo-oligoKdo production in strain 1021.

Structural characterization of polysaccharides.

Three different media (YTA, TY, and YEM) were used for the propagation of bacteria to detect their possible influence on polysaccharide production. Strain AK631, the *exoB* mutant of *S. meliloti* 41, was conventionally used as background for the introduction of single and double mutations. As the crude extract analysis demonstrated, this parent strain produced an abundant amount of (GlcA-Pse)_n oligo and polymers and a significant amount of lipo-oligoKdos (especially in TY medium) and glycerophosphate-substituted regular glucans (Fig. 4), as was reported recently (Chataigne et al. 2008). After mild hydrolysis of the AK631 polysaccharide crude extract, the KPS repetitive unit and Kdo were detected in significant quantities, as was the KPS lipid anchor (m/z 622.48 in the negative mode). In the *rkpY*₃₂₂₂ mutant, neither (GlcA-Pse)_n nor lipo-oligoKdo could be detected. Only free oligoKdo and an important amount of glycerophosphorylated glucans could be observed (Fig. 4). The

proportion and amount of these latter two components were dependent on the medium. After mild hydrolysis, no KPS lipid anchor could be detected. The *rkpZ/rkpY* double mutant produced only glycerophosphorylated glucans (Table 2).

When *S. meliloti* 1021 harbored the *rkpY* gene from strain 41, it produced dramatically reduced lipo-oligoKdo compared with that of the parent strain. No lipid anchor (m/z 622.48) could be detected in the crude extract or after mild hydrolysis. Depending on the medium used for propagation, free oligoKdo (without anchor) could be detected in various amounts (Table 2). This result confirmed our observation in DOC-PAGE experiments that the presence of the *rkpY* gene in strain 1021 had a negative effect on the lipo-oligoKdo production.

DISCUSSION

Despite the growing body of evidence that KPS play an essential role in many plant-microbe interactions, there are relatively few data highlighting their importance in the invasion of nitrogen-fixing symbiotic nodules (Frayse et al. 2003; Jones et al. 2007). Earlier, we identified several genes in the *rkp-3* region of *S. meliloti* 41 (Kiss et al. 2001) that are respon-

Fig. 4. Structural characterization of polysaccharides produced by the *rkpY* mutants. Electrospray ionization/quadrupole time-of-flight mass spectrometry (TOF MS ES) spectra of the **1**, crude extract with in source energy; **2**, saccharide fraction after mild hydrolysis; and **3**, lipid fraction after mild hydrolysis for **A**, NE3222 and **B**, AK631. Ions at m/z 359, 535 (553), and 1,070 are characteristic for the high molecular weight capsular polysaccharides (KPS) (K_R5). M/z 439 and 659 are signatures for oligo 3-deoxy-d-manno-2 octulosonic acid (Kdo) and 622 for the lipo-oligoKdo anchor (KPS anchor). Ions at m/z 981, 1,143, and 1,342 correspond to the glycerophosphorylated glycans (gGly).

Table 2. Chemical analysis of polysaccharide production of representative mutants^a

Strain	Genotype	GlcA-Pse	Lipo oligoKdo	Free oligoKdo	Free anchor	β-Glucans
AK631	Sm41 <i>exoB</i>	++++	++	+	+	+
NE3222	<i>rkpY</i> ₃₂₂₂	—	—	++ ^b	—	++ ^b
PP4180	<i>rkpZ</i> ₄₁₈₀	+++	+	+	+	++
AV476	<i>rkpY</i> ₃₂₂₂ , <i>rkpZ</i> ::ET-KanR-3	—	—	(—)	—	+++ ^b
Sm1021	<i>Sinorhizobium meliloti</i> 1021 wild type	—	++++	+++	+	+
AV4517	<i>S. meliloti</i> 1021 (<i>rkpY</i> ⁺)	—	—	+ ^b	—	+

^a GlcA, glucuronic acid; Pse, pseudaminic acid; KDO, 3-deoxy-d-manno-2 octulosonic acid; —, not detectable; +, less than 10%; ++, approximately 40%; +++, approximately 70% of the maximum amount detected.

^b Amount of polysaccharide depends on the medium used. Data shown were obtained using cells cultivated in YTA medium (Orosz et al. 1973) and harvested in the same growth phase.

sible for the production of the K_R5 antigen. This strain-specific KPS consists of disaccharide repeating units composed of GlcA and Pse (Reuhs et al. 1993, 1998). The *rkpLMNOPQ* gene cluster in the *rkp-3* region was suggested to be involved in Pse biosynthesis, one of the component of the disaccharide subunit. In this work, we extended the DNA sequence determination of the *rkp-3* region and presented the results of a genetic analysis, the aim of which was to clarify the possible function of the newly identified ORF.

On the basis of the mutant phenotypes, only one ORF, *rkpY*, could be identified as an essential element of the K_R5 antigen production, of the infection process in symbiosis, and of the infection of phage 16-3. Single-insertion mutations in *orf7343*, *orf8077*, and *rkpT2* did not affect the above functions, suggesting that these putative genes are either not necessary or not essential for these events.

The putative RkpY protein does not possess any known motifs or domains except some transmembrane domains. The number of these predicted domains varies between 0 to 4, depending on the program and the settings applied. To detect possible periplasmic segments of RkpY and other proteins encoded in the *rkp-3* region, we isolated translational gene fusions using *TnphoA* (Manoil et al. 1990; Beckwith 2000). As expected, active fusions in *rkpM*, *rkpR*, and *rkpT2* could be successfully isolated. The three fusions localized in the middle of the *rkpR* gene support one of the topology predictions, where an N-terminal and a C-terminal transmembrane domain (residues 23 to 43 and 319 to 339) border a 275-aa-long extracellular domain (in which the fusions were localized) (Fig. 2).

A similar structure harboring a 616-aa-long extracellular domain (1,848 bp at DNA level) was also predicted for RkpY. However, we could not isolate any active fusion in the *rkpY* gene supporting this model. On the basis of this negative result, one may speculate that i) RkpY is not a membrane protein or, what seems to be more probable, ii) RkpY has only one transmembrane domain that was predicted at the very end of the protein (residues 978 to 998) (Fig. 2). Unfortunately, this latter topology makes the isolation of active gene fusions by *TnphoA* impossible, because the existence of an N-terminal transmembrane domain that directs the C-terminal fusion product to the periplasm is a prerequisite for the formation of active alkaline phosphatase (Manoil et al. 1990).

Other results presented here, however, indicate that RkpY is a membrane protein. We have identified a special missense mutation in allele *rkpYL552P* that, in contrast to several other mutations in this gene, made the isolation of host range phage mutants possible. These host range phage mutants carry a missense mutation in gene *h* (V. Deak, *unpublished results*), that probably encodes the tail fiber protein (Putnoky et al. 2004). These results suggest that residue L552 of RkpY is directly involved in the phage recognition or infection process. That is, RkpY functions as a membrane protein. It may possess only one C-terminal transmembrane domain that orient the 977-aa-long N-terminal part outside (Fig. 2). Our recent data indicate that RkpY is associated with RkpM and RkpZ, because these proteins were also shown to be involved in phage 16-3 adsorption or infection process (Putnoky et al. 2004).

This study presents further evidence that RkpM is also a membrane protein. Because active *phoA* fusions were identified after position 70 and 105 residues, only the first of the two predicted transmembrane domains (residues 50 to 70 and 123 to 143) may exist that orients the C-terminal part outside (Fig. 2).

One of the basic questions is the function of the *rkpY* gene. The *rkpY* mutants produce an LMW, Alcian-blue-staining material that was supposed to be an LMW derivative of the K_R5 antigen. Here, we have presented genetic evidence that this idea was wrong, because many essential biosynthesis genes of

the K_R5 antigen (*rkpK*, *rkpL*, *rkpM*, and *rkpQ*) proved to be unnecessary for the production of this LMW PS. Polysaccharide analysis of different mutant strains supported the results of the genetic experiments. No K_R5 antigen, (GlcA-Pse)_n, was detected in the polysaccharide samples of an *rkpY* mutant. Moreover, both the defect of *rkpY* in strain 41 and the presence of the wild-type gene in strain 1021 disturbed the synthesis of lipo-oligoKdo, suggesting a possible role for RkpY in the lipo-oligoKdo production.

Previous studies indicated that *rkpZ1* mutation in strain 1021 destroyed the Kdo homopolymer production (Sharypova et al. 2006). In *rkpZ/rkpY* double mutants of strain 41, neither lipo-oligoKdo nor free oligoKdo were detected. This result suggests that the LMW PS visible in the DOC-PAGE of the *rkpY* mutants (Fig. 3C, D, and E) is probably oligoKdo.

Results presented here show that the *rkpY* gene is involved in the K_R5 antigen as well as in the lipo-oligoKdo production and that it influences the amount of β -glucans presenting a pleiotropic effect on the polysaccharide production of the cells. To determine its exact function, further studies will be performed.

Two recent works have underlined the importance of KPS in symbiosis. A complete *rkp-3* gene set except *rkpP* was described in *Rhizobium* sp. strain NGR234. These genes are also necessary for the biosynthesis of a K-antigen, consisting of disaccharide repeating units of GlcA and Pse. It has a very similar structure to that of the K_R5 antigen of *S. meliloti* 41 (Le Quere et al. 2006). Deletion mutant *NGR Δ rkpMNO* shows a reduced nodulation efficiency in different host plants, although the mutant bacteria could establish nitrogen-fixing symbiosis. The presence of an *rkpY* homologue sequence was also reported but its importance in KPS production and symbiosis has not yet been investigated.

Another rhizobia, *S. fredii* HH103, produces a Pse homopolymer (Gil-Serrano et al. 1999) that is essential for symbiosis, as was shown by *rkp-1* region mutants (Parada et al. 2006). Although the presence of the *rkpLMNOPQ* gene cluster has not been reported, one may predict that a homologue gene cluster should be present in *S. fredii* HH103, similar to those bacteria that produce Pse.

A Pse biosynthesis pathway and the corresponding *pse* genes have been studied more in detail in *Campilobacter* and *Helicobacter* spp. (Schirm et al. 2003; Guerry et al. 2006). Interestingly, in these bacteria, the *pse* genes do not form one cluster although they located close to each other in the same chromosomal region. In rhizobia and in several other bacteria (as we found in the in silico analysis), the corresponding genes (*rkpLMNO[P]Q*) form one cluster. Interestingly, *rkpY* is not a fundamental element of these clusters. It was identified only in *S. meliloti* 41 and in *Rhizobium* sp. strain NGR234, that produces a structurally very similar Pse containing K-antigen. These strains provide good backgrounds to study how RkpY protein influences polysaccharide production.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, plasmids, and growth conditions.

Rhizobium strains and plasmids used in this study are shown in Table 1 and Figure 1. *E. coli* strains XL1-Blue and DH5 α were used for transformation (Sambrook et al. 1989). For cloning and sequencing, plasmids pBluescriptII SK(+) (Stratagene, La Jolla, CA, U.S.A.), pUC19 (Yanisch-Perron et al. 1985), pBBR1-MCS2 and pBBR1-MCS5 (Kovach et al. 1995), and pPAG160 (Ganyu et al. 2005) were applied. Media, antibiotic concentrations, culture conditions for *E. coli*, and *S. meliloti* strains and phage 16-3 sensitivity tests have been described previously (Putnoky and Kondorosi 1986; Putnoky et al. 1990).

Identification of spontaneous *rkpY* and host range phage 16-3 mutants.

We applied phage 16-3 as a selection tool for the isolation of *rkp* mutants, as described earlier (Kereszt et al. 1998). Strains producing a KPS pattern in DOC-PAGE similar to that of *rkpY*::Tn5 mutants (Fig. 3C) were chosen for genetic complementation experiments. In all of these strains, K_R5 antigen production and phage sensitivity were restored by the introduction of the wild-type allele of *rkpY* (Fig. 1C, pNE3265), supporting the idea that they have the defect in the *rkpY* gene. A representative mutant, NE3222, carrying *rkpY*₃₂₂₂ allele was chosen for the construction of the double mutants.

Mutant bacteria, resistant to wild-type phage 16-3 but sensitive to host range phage mutants, were isolated as described recently (Putnoky et al. 2004). An *rkpY* mutant strain, PP4073, was identified by the help of DOC-PAGE analysis and complementation experiments as described above. Three subclones of the *rkpY* gene (pAV568, pAV571, and pAV579) (Fig. 1C) were used in marker rescue experiments to delimit the mutation in PP4073. The nucleotide sequence of the mutant allele was established after isolating the appropriate DNA-region by PCR (discussed above).

Transposon mutagenesis and homologous recombinants.

Random transposon Tn*phoA* insertions were generated on cosmid clone pAT401, as described previously (Putnoky et al. 1998). Insertions resulting in alkaline phosphatase activity in *S. meliloti* 41 were selected for sequence determination. The exact positions of the insertions were established by a *phoA*-specific reverse primer (GCAGTAATATCGCCCTGAGCAG) after subcloning the target sequence together with the kanamycin-resistant 5' end of Tn*phoA*.

An in vitro transposon mutagenesis on plasmids pAV433, pPP2543, pPP4166, and pPP4170 (Fig. 1C) was carried out by the ET-KanR-3 Mu Entranceposon (TGS II kit; Finnzymes, Espoo, Finland) according to the instruction manual. Positions of the insertions were determined first by restriction mapping; then, some of them were chosen to establish the DNA sequence of the target region. Insertions localized in *rkp* genes (Fig. 1B) were recloned with the target region into pPAG160 (Ganyu et al. 2005) for direct mutagenesis of the genomic sequence. Plasmid pPAG160 carries spectinomycin/streptomycin resistance (Sp^r/Str^r), a pCU1 *mob* region, and a pSC101 replication origin (Thatte et al. 1985). It can be mobilized by pCU101 helper plasmid but it is unable to replicate in *S. meliloti*. Not only homologous recombination but also coinfectant formation and additional recombination events may result in kanamycin-resistant derivatives; therefore, kanamycin- and spectinomycin-sensitive transconjugants were tested for the presence of plasmid sequences by PCR using oligonucleotide primer pairs designed for the pSC101 region (GCTTGCGAGGGTGCTA CTTA, CGGTTACGAGATCCATTGCT) and for the Ω fragment (Sp^r/Str^r) (GCGCGATTTTGCCGGTTACT, GATGTTAC GCAGCAGGGCAGTC). In addition, locations of the insertions in the targeted genes were also confirmed by PCR. Recombinants harboring neither plasmid regions were chosen for further work. Representative Tn5 insertions in different *rkp* genes (Table 1) were introduced on pLAFR1 cosmid clones into the AK631 genome via homologous recombination as described earlier (Kiss et al. 2001).

DNA manipulations and sequence determination.

Standard procedures, including DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, DNA ligation, transformation, radioactive labeling, and Southern hybridization were performed using conventional methods (Sambrook et al. 1989) or as recommended by the suppliers. For sequence deter-

mination, appropriate restriction fragments were subcloned into the vectors pUC19 or pBluescript II SK(+) and their nucleotide sequences were determined by the BigDyeTerminator kit on an Applied Biosystems 373A sequencer. Additional subsequences were obtained from Tn5-flanking regions of different insertional mutants using a Tn5-specific oligonucleotide primer (GCAAAA CGGGAAAGGTTCCG), and from ET-KanR-3 insertions using SeqE or SeqW primers (TGS II kit). The nucleotide sequence of the *rkpY*₄₀₇₃ allele was established after amplification with primer pair Y-Bam734U (AGCGAAATAGCGGGATGTTC) and Y-Bam2163L (GCCGGAATCCGTTTCTGA). For sequence determination, the above primers and two additional ones, Y-Bam1375U (TTGCTAGCCGCTCCCGA) and Y-Bam1531L (CCTGGAGGGGCTGGTCTTT), were used.

DNA sequences were determined in at least two independent reactions at both strands. First, a 5,966-bp sequence of the *rkpY* region has been registered in the EMBL, GenBank, and DDBJ Nucleotide Sequence Databases under accession number AJ249130. Later, this sequence was extended in both directions, resulting in a 12,289-bp region and a new record (AM849044) that overlaps in 86 bp with the sequence record of the *rkpL-rkpZ* region (AJ245666) published earlier (Kiss et al. 2001).

Computer-assisted sequence analysis.

PCGene software (designed by Amos Bairoch; Intelligenetics Corp., Mountain View, CA, U.S.A.) and the GCG program package (Devereux et al. 1984) were used for basic DNA sequence analysis and assembly. Amino acid homology searches were performed against the nonredundant database of the NCBI BLAST Network Service with the BlastP program. Protein family and domain predictions were performed with different proteomics tools from the ExPASy Molecular Biology Server. Transmembrane domains were calculated with the following programs: TopPred2 and DAS at the Stockholm Bioinformatics Center and SPLIT 4.0 at the University of Split. The codon preference program of the GCG software package was used to identify ORF showing codon usage similar to that of an average housekeeping *S. meliloti* gene as described previously (Kiss et al. 2001).

We performed a search for the homologues of the *rkp-3* genes. First, BLASTP searches against the trEMBL database were carried out using the predicted Rkp protein sequences. When a homologue sequence was identified, the corresponding DNA region was examined for the presence of the whole gene cluster. Overall similarity to a given homologue was higher than 60%.

DOC-PAGE analysis of polysaccharides.

Extraction of surface polysaccharides by a modified hot phenol-water method and DOC-PAGE analysis were carried out as described previously (Reuhs et al. 1993; Kiss et al. 1997). The phenol-water-extracted materials from bacteria were separated in 18% DOC-PAGE and were visualized using Alcian blue silver staining. For KPS-specific analysis, the gels were stained without oxidation (Fig. 3); for LPS-specific staining, no Alcian blue was applied and sodium meta-periodate was used as oxidizer.

Purification of LPS and KPS for chemical analysis.

For chemical analyses, bacteria were cultured at 28°C in YTA (Orosz et al. 1973), TY (Beringer 1974), or YEM (Vincent 1970) medium. The cells were pelleted at early log phase, then washed by resuspending in deionized water followed by centrifugation. Polysaccharides were purified from representative mutants and were analyzed as described recently (Chataigne et al. 2008). Briefly, samples were extracted by the hot-phenol

water method. The aqueous supernatant was dialyzed five times at 1:100 in water adjusted to pH 7.8 with NH_4OH (to minimize Na^+ and Ca^{2+} associations and optimize solubilization) using a 12,000-kDa MWCO membrane. After the complete elimination of phenol traces, the water phases were made 10 mM in MgSO_4 and 50 mM in Tris-HCl (pH 7.0) and incubated with RNase and DNase for 48 h at 5°C. Finally, proteinase K treatment was applied at 37°C for 4 h. The sample were dialyzed again and freeze dried.

Preparation of the saccharide and lipid part of the crude extracts.

To release lipid anchor moieties from lipidated polysaccharides (lipidated oligoKdo and LPS), samples of the crude extracts (1 mg/ml) were treated with 1% acetic acid for 1 h at 100°C. Then, the samples were centrifuged and the aqueous phases were extracted using chloroform in a 1:3 (vol/vol) ratio to eliminate residual lipid anchor. The samples were evaporated at 40°C under a stream of filtered nitrogen.

Electrospray ionization/quadrupole time-of-flight mass spectrometry analysis.

Electrospray ionization/quadrupole time-of-flight (Q-tof) mass spectrometry (MS) analysis was performed in the negative-ion mode using a Q-tof Ultima Instrument (Waters, Milford, MA, U.S.A.).

Crude extract analysis. Samples were dissolved at a concentration of approximately 10 ng/ μl in a 50:50:0.001 (vol/vol/vol) mixture of 2-propanol, water, and triethylamine and sprayed at a flow rate of 10 $\mu\text{l}/\text{min}$. Capillary entrance voltage was set to 3.0 kV and temperatures of source and dry gas were set to 100 and 120°C, respectively. Settings were as follows: cone: 60 V; Rf lens: 25 V; and MS profile = m/z 150 (time 20%), m/z 900 (time 60%), and ramping 20%.

Anchor analysis. Samples were dissolved at a concentration of approximately 50 ng/ μl in a $\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O}$ (60:30:5) adjusted to 5 mM in triethylamine. MS conditions are similar to the previous one, except that Cone voltage was 100 V and Rf lens was 70 V.

The spectra, which showed several charge states for each component, were charge deconvoluted, and mass numbers were given refer to the monoisotopic molecular masses using the MaxEnt 3 software (MassLynx, Waters).

Mono and oligosaccharide analysis. Samples were dissolved at a concentration of 10 ng/ μl in $\text{MeOH}/\text{H}_2\text{O}$ (1:1) adjusted to 5 mM in triethylamine. MS conditions were the same as for the crude extracts.

Plant tests.

Assays for the symbiotic properties of the mutant *Rhizobium meliloti* strains were carried out with alfalfa (*Medicago sativa* L. Nagyszénási) plantlets, as previously described (Putnoky et al. 1988).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

NCBI BLAST Network Service: www.ncbi.nlm.nih.gov/blast/Blast.cgi
 ExPASy Molecular Biology Server: www.expasy.org
 Stockholm Bioinformatics Center: www.sbc.su.se/services
 University of Split: split.pmfst.hr/split/4