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The capsular polysaccharide biosynthesis and *16-3* bacteriophage receptor in *Sinorhizobium meliloti* 41

Ph.D. thesis

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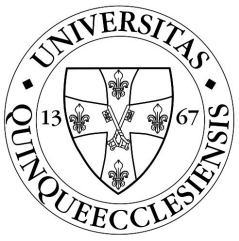

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INTRODUCTION

Symbiosis between alfalfa (*Medicago sativa*) and *Sinorhizobium meliloti* bacteria that belongs to rhizobia is an important model of plant-microbe interaction and nitrogen fixing symbiosis. In order to better understand the development and functioning of this symbiosis it is important to identify and study the bacterial genes that participate in this process. Bacterial surface capsular polysaccharides are important factors in the development of symbiosis and they may block the infection of bacteriophages. Our aim was to study the role of capsular polysaccharide of *S. meliloti* 41 in symbiotic plant-bacterium and bacterium-bacteriophage recognition using mainly genetic tools.

With the help of endosymbiotic *S. meliloti* bacteria, the host plants (alfalfa, sweet clover, fenugreek) are able to get an almost inexhaustible nitrogen source, the atmospheric dinitrogen. Development of symbiosis is based on a complex molecular signal exchange in which the fine regulation of the expression of several bacterial and plant genes is necessary. Rhizobia live in the soil where they are able to sense flavonoids secreted by the root of their host legume plant. Flavonoids trigger the secretion of Nod-factors, which in turn are recognized by the host plant and can lead to root hair deformation and to the development of nodule. Bacteria infect the root via the infection thread that begins to grow from the root hair toward to the developing nodule cells. In the course of invasion bacteria enter the nodule cells where they differentiate morphologically into bacteroids and begin to reduce dinitrogen to ammonium by the enzyme nitrogenase.

Cell-surface polysaccharides of rhizobia are involved in several steps of nodulation process, such as infection thread initiation and development, nodule invasion, and host specificity. Like several gram-negative bacteria, *S. meliloti* produces various mucoid polysaccharides. Study of several infection defective (Inf$^-$) bacterial mutant elicits the role of these polysaccharides in symbiosis. The exopolysaccharides (EPS) are secreted to the external environment and may suppress the plant defence reactions. The lipopolysaccharide (LPS) is relevant in the latter phase of invasion, while the capsular polysaccharide (KPS, K-antigen) is located around the cell membrane and may play a role in the early phase of invasion.

Numerous *S. meliloti* mutants are not able to infect the host plant due to a defect in EPS production. However, other strains like the *exo* mutants of *S. meliloti* 41 are able to establish symbiosis. The reason of this contradiction is that *S. meliloti* 41 produces a strain-specific capsular polysaccharide, the K_R5 antigen, which functionally compensates for the EPS I (succinoglycan) during the infection process. Recently, the structure and genetics of this antigen were mainly elucidated. It is composed of a low- and a high-molecular-weight (LMW, HMW)

polysaccharide containing glucuronic acid (GlcA) and pseudaminic acid (Pse) disaccharide subunits which are decorated by 5-N-β-hydroxybutyryl and 7-N-acetyl residues ([(5-OHBut, 7-*N*AcPse)-GlcA]). Three *rkp* gene clusters (*rkp-1*, *rkp-2* and *rkp-3*) were shown to be involved in the K_R5 antigen biosynthesis.

The *rkp-1* region harbors genes (*rkpABCDEF*) responsible for the synthesis of a specific lipid carrier for KPS biosynthesis, and genes (*rkpG-J*) involved in the modification and transfer of the carrier's lipophilic molecules. In this region, there are no genes involved in the biosynthesis of the sugar components or in the polymerization process.

The *rkp-2* region carries two genes (*lpsL, rkpK*), both necessary for wild-type LPS production. Moreover, *rkpK* was shown to be required for the synthesis of a K_R5 antigen precursor sugar (UDP-glucuronic acid) by encoding a UDP-glucose dehydrogenase.

The above mentioned two regions are common in the *Sinorhizobium* genus but the *rkp-3* region was identified only in strain *S. meliloti* 41. The *rkp-3* region lies on the pRme41c megaplasmid (pSymB) and contains several polysaccharide biosynthesis genes. The *rkpLMNOPQ* gene cluster presumably performs the synthesis of the pseudaminic acid (Pse) component of the K_R5 antigen. Three additional genes (*rkpR, rkpS and rkpT*) encode for putative products with 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 encodes for RkpZ that decreases the chain length to the production of LMW KPS. Outside but close to this region was identified the *rkpY* gene of which product has no any similarity to any known proteins. Mutants in this gene lack K_R5 antigen and produce an unknown LMW polysaccharide (LMW PS).

In the study of cell surface structures and in the identification of their biosynthesis genes strain specific bacteriophages proved to be important tools. In the study of the K_R5 antigen, bacteriophage *16-3* that is a temperate, specialized transducing phage of *S. meliloti* strain 41 was essential.

The Inf- mutants mentioned above are not only defective in KPS production but also possess resistance against bacteriophage *16-3*. This result suggests that the presence of phage receptor and the polysaccharide biosynthesis are related. It was supposed that the K_R5 antigen is the phage receptor. This fact serve a possibility to know more in detail both the KPS biosynthesis genes and the components of the phage receptor by isolating bacterium mutants presenting altered receptor structure.

AIMS

The main aims of the present study were:

1) to know more in detail the K_R5-antigen biosynthesis for characterization of plant-bacteria and bacteria-phage recognition processes,
2) to extend and analyze the sequence of the *rkpY* region,
3) to characterize the *rkpY* and the newly identified genes in the *rkp-3* region by genetic and biochemical experiments.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, plasmids, and growth conditions

In the course of the work we used and constructed several bacterial and bacteriophage strains. The *S. meliloti* derivatives were propagated on complete TA and minimal GTS media at 28°C. The *Escherichia coli* strains were propagated on LB media at 37°C. The appropriate antibiotic was applied to control the strains or to select for transconjugants or transformants. *E. coli* strains XL1-Blue and DH5α were used for transformation. For cloning and sequencing plasmids pBluescriptII SK(+), pUC19, pBBR1-MCS2, pBBR1-MCS5 and pPAG160 were applied. For propagation of *16-3* phage *S. meliloti* strain 41 (RM41) was used. The phage titer was 10^9-10^{10} PFU/ml in the phage lysates.

Isolation of phage receptor mutants and identification of the mutant genes

We applied phage *16-3* as a selection tool for the isolation of the mutants. Among the phage resistant mutants we screened for those bacteria that presented an altered receptor unable to adsorb the wild-type phage but able to adsorb host range phage mutants. Host range phages carry mutations in genes that encode for tail proteins necessary for the host recognition.

We determined the position of the bacterial mutations by complementation experiments. Cosmid clones carrying either wild-type or mutant *rkp* regions with different Tn*5* insertions were introduced by conjugation into the receptor mutants and the transconjugant strains were tested for phage-resistance by the wild-type *16-3* phage. When a wild-type cosmid clone was able to restore the phage sensitivity the wild-type allele of the mutant gene was considered to lay in the region introduced. When a Tn5 insertion on a complementing cosmid blocked the complementation and the transconjugant bacteria remained *16-3* phage resistant, it was supposed that the cosmid carries the Tn5 insertion in the same gene as the receptor mutant strain.

We determined the position and the nature of the mutation in the $rkpM_{4046}$ allele by DNA sequencing. Total DNA were isolated from the mutant bacteria with phenol-chloroform method.

After amplifying the gene segments by polymerase chain reactions (PCR) the DNA fragments were isolated and their sequence were determined.

The mutation in the *rkpY*$_{4073}$ allele was localized first by marker rescue experiments. We took advantage of our observation that phage *M1* of *S. meliloti* 1021 could infect the *rkp* mutants of *S. meliloti* 41. Therefore, we examined which subfragment of the *rkpY* gene generates phage *M1* resistant wild-type bacteria by homologous recombination. Three overlapping subclones of the wild-type gene were introduced by conjugation to the mutant strain and the transconjugant populations were screened for the appearance of wild-type homologous recombinants by phage M1. The nucleotide sequence of the mutant allele was established after isolating the appropriate DNA-region by PCR.

Extension of the nucleotide sequence in the *rkp-3* region

For sequence determination, appropriate restriction fragments of plasmids pAT4101 and pPP2543 containing segments of the *rkp-3* region were subcloned into pBluescript II SK(+) vector. Sequencing reactions were carried out by the BigDyeTerminator Kit using T7 and T3 primers and the sequences were determined on an Applied Biosystems 373A sequencer. Additional subsequences were obtained from Tn*5*-flanking regions of different insertional mutants using a Tn*5*-specific oligonucleotide primer, and from ET-KanR-3 insertions using SeqE or SeqW primers. DNA sequences were determined in at least two independent reactions at both strands. The extended sequence has been registered in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under accession number AM849044.

Amino acid homology searches were performed against the nonredundant database of the NCBI BLAST Network Service with the BlastP program (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Protein family and domain predictions were performed with different proteomics tools from the ExPASy Molecular Biology Server (http://www.expasy.org/). Transmembrane domains were calculated with the following programs: TopPred2 and DAS at the Stockholm Bioinformatics Center; (http://www.sbc.su.se/services/); SPLIT 4.0 at the University of Split (http://split.pmfst.hr/split/4/).

Mutagenesis of the *rkpY*-region and construction of mutant bacteria

Mutant strains defective in a putative gene in the *rkpY* region were generated by the help of transposon mutagenesis. Cosmid pAT4101 and different subclones that contained genes from the right part of the *rkp-3* region, was mutagenized *in vitro* by the ET-KanR-3 Mu Entranceposon (TGS II Kit, Finnzymes, Fi) according to the instruction manual. The insertion were localized by physical mapping and their positions were verified by sequence determination using the SeqE

and SeqW primers. DNA fragments carrying mutations in different open reading frames (ORF) were built into the conjugative pPAG160 vector. Single and double bacteria mutants were established by conjugation of pPAG160 derivatives into *S. meliloti* 41 or into the its different *rkp* mutant versions. The existence of cointegrate and homologue recombinants were examined by PCR using primers for the vector sequences and for the target sequence.

Deoxycholate-polyacrylamide gel electrophoresis (DOC-PAGE)

Isolation of the surface polysaccharides (KPS, LPS, polyKdo) were carried out by a modified hot phenol-water extraction method. The samples were dialyzed against distillated water and were dried by lyophilization, finally were solved in distillated water. The phenol-water extracted materials from bacteria were separated in 18% DOC-PAGE (BioRad Miniprotean II) and were visualized using Alcian blue silver staining. For KPS-specific analysis, the gels were stained without oxidation, for LPS-specific staining no Alcian blue was applied and sodium meta-periodate was used as oxidizer. Photos were taken by a UVP BioDoc-It System.

Plant test

Assays for the symbiotic properties of the mutant *S. meliloti* strains were carried out using alfalfa (*Medicago sativa* L. Nagyszénási) seedlings. Steril alfalfa seeds were germinated on nitrogen free medium in test tubes, and the 4 to 6 days old seedlings were infected by the mutant strains. Evaluation of the experiments were carried out 4 to 6 weeks after infection.

RESULTS AND DISCUSSION

Our previous results suggested that the K_R5 antigen biosynthesis and the presence of the phage receptor are connected to each other. We supposed that the study of the receptor defective mutants identified by host range phages would help to discover novel biosynthesis genes. As a first step in this work, spontaneous mutations were isolated in *S. meliloti* 41 (strains GH4046, GH4178, GH4180 and PP4073) that blocked the infection of the wild-type *16-3* phage but which made the isolation of host-range phage mutants possible. We supposed that in these cases an altered phage receptor is present on the cell surface that allows only the infection of those mutant phages that possess a host range mutation in a tail fiber gene. In the further work, we aimed to localize the special receptor mutations and determine their impact on the cell surface structures.

Phage *16-3* receptor has protein constituents

As we expected, DOC-PAGE experiments showed that the new phage resistant mutants were affected in the K_R5 antigen production. In order to decide which *rkp* gene harbors the mutation in the receptor mutant strains, genetic complementation experiments were carried out by introducing the *rkp-1*, *rkp-2*, and *rkp-3* gene regions on different cosmid clones, separately.

Representative cosmid clones of the *rkp-3* region could complement all of the mutations suggesting that they are located in previously identified genes. In further complementation experiments, where different mutant derivatives of the region were introduced into the mutants, we could manage to identify the mutant genes. According to the results, the mutations were localized in genes *rkpM* (GH4046), r*kpZ* (GH4180 and GH4178), and *rkpY* (PP4073).

The DOC-PAGE analysis of surface polysaccharides of the $rkpM_{4046}$ mutant gave a surprising result. Earlier we presumed that the new mutants will produce an altered KPS lacking hydroxybutyryl and/or acetyl modifications but the mutant bacteria produce no K_R5 antigen at al. This result indicated that not the K_R5 antigen is the phage receptor, since we have shown by the isolation of host range phage mutants that $rkpM_{4046}$ mutant has an altered receptor. Interestingly, *rkpM* mutants carrying Tn5 insertions were not suitable for the isolation of host range phages indicating that the receptor is not present on these mutants. These results suggested that $rkpM_{4046}$ is a special allele.

Analysis of the $rkpY_{4073}$ mutation presented the same result. Several spontaneous and Tn5 mutations in the *rkpY* gene resulted in the lack of the phage receptor. Only the $rkpY_{4073}$ mutant was suitable for the isolation of host range phages suggesting, that a modified receptor is presented on the bacterial cell surface. Interestingly, *rkpY* mutant strains produce a low-molecular-mass polysaccharide (LMW PS) instead of K_R5 antigen.

Because of the special features of the above mutations, DNA sequences of the mutant alleles were determined. In both mutants, one missense mutation was found resulting a Leu_{252} to Phe_{252} substitution in $rkpM_{4046}$ allele and a Leu_{552} to Pro_{552} substitution in $rkpY_{4073}$ allele, respectively. In the host range phage mutants, we could also localize different missense mutations in two phage genes suggesting a direct protein-protein contact between the putative tail fiber proteins of the phage and the K_R5 antigen biosynthesis proteins of *S. meliloti* 41. Therefore, we suggest that the RkpM and the RkpY proteins take part in the formation of the phage *16-3* receptor.

In case of RkpZ protein, we could not draw this conclusion, because on all *rkpZ* mutants including Tn5 insertion mutants was possible to isolate host-range phages.

In the literature, protein type bacteriophage receptors are well known. Several missense mutations of the *malB* (*lamB*) gene of *E. coli* K-12 were described that blocked the recognition of lambda phage and allowed the selection for host range mutants. These lambda mutants carried different missense mutations in gene J encoding for the tail fiber protein. The K phages (capsule-specific phages) attach in two steps to the *E. coli* cell surface. First, the bacteriophage adsorbs to the capsular polysaccharide and degrades it by enzymatic way. As a next step, it recognizes a channel protein that helps to allocate its DNA into the cell. According to our result, in case of *S. meliloti* 41 and phage *16-3* a similar two-step recognition process cannot be excluded. The

RkpM and RkpY proteins, as a part of the K_R5 antigen biosynthesis apparatus, may have a direct role in phage adsorption. However, the role of RkpZ may be indirect, and additional analysis is necessary to understand. It remains to clarify whether the HMW KPS produced by the rkpZ mutants or the lack of RkpZ protein is the reason of the modified phage adsorption.

Sequence analysis of the *rkpY* region

Recently, a 12821 bp sequence of the *rkp-3* region has been determined, that covers the *rkpLMNPOQ*, *rkpRST* and *rkpZ* genes. An additional 5966 bp long DNA segment carrying the *rkpY* gene was also established earlier. To complete the nucleotide sequence of the *rkp-3* region additional subsequences were determined that covered the gap between the two sequences and extended the sequence to the "right" part of the *rkpY* gene. The extended nucleotide sequence of the *rkp-3* region is 25024 bps long. In this sequence, further putative genes were identified in both side of the *rkpY* gene. On the basis of computational analysis, several open reading frames (ORFs) were found between the *rkpZ* and *rkpY* genes that show homology to different transposon genes. These are the components of two insertional elements (ISRm26, ISRm15), that probably inserted into the *rkp-3* region by two independent events. Sequence analysis suggested that first ISRm15 has inserted between the *rkpZ* and *rkpY* genes, later the ISRm26 was integrated into ISRm15 sequence.

On the "right" side of *rkpY,* three additional ORFs were identified: *orf7343*, *orf8077*, and *rkpT2*. In our further work, we wanted to determine whether these putative genes have any function in KPS production or in symbiosis. To answer these questions we decided to create several single and double bacterial mutants.

Genetic analysis of the *rkpY* region

Using transposon mutagenesis mutations were generated in *orf7343*, *orf8077* and *rkpT2*. Mutant bacteria were examined for phage resistance and symbiotic activity and polysaccharides produced by these strains were analyzed with DOC-PAGE. According to our results *orf7343*, *orf8077* and *rkpT2* have no direct function in either polysaccharide production, symbiosis or the propagation of phage *16-3*. We also wanted to examine whether these ORFs and other known *rkp* genes are involved in the synthesis of the unknown LMW polysaccharide (LMW PS) produced by the *rkpY* mutants. To answer this question we decided to introduce several *rkp* mutations into *rkpY* mutant backgrounds.

Mutation in *rkpZ* affects the LMW polysaccharide production in *rkpY* mutants

We have introduced a second mutation into representative genes of the *rkp-1*, *rkp-2*, and *rkp-3* regions using an *rkpY* mutant background. We supposed earlier, that the LMW PS produced by the *rkpY* mutant is a kind of precursor of the K_R5 antigen. If it is correct, several double mutants

should fail to produce this material. Interestingly, the second mutations, except in the *rkpZ* gene, did not influence the LMW PS production of the *rkpY* mutant, suggesting that the *rkp* genes, which are otherwise essential for K_R5 antigen production, are not involved in the biosynthesis of the LMW PS. That is the LMW PS is not composed from pseudaminic acid and/or glucuronic acid.

In contrast, the *rkpZ*/*rkpY* mutant strain failed to produce any detectable amount of LMW PS, suggesting that the *rkpZ* gene influences the synthesis of this unknown polysaccharide. In *S. meliloti* strain 41, the only *rkpZ* gene influences the polymerization of the K_R5-antigen. However, in strain 1021 there are two paralogous *rkpZ* genes that play a role in the Kdo homopolymer (oligoKdo) biosynthesis. Therefore, we presumed that 1) the LMW PS produced by the *rkpY* mutants of strain 41 is oligoKdo, and 2) the function of RkpY in strain 41 is to suppress the oligoKdo synthesis (that does not function in *rkpY* mutants).

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

In the genom sequence of *S. meliloti* 1021, we could not detect any *rkpY* related gene. To test how the presence of *rkpY* gene influences the polysaccharide production of this strain, it was introduced into the wild-type background. For this purpose, the wild-type *rkpY* gene was cloned into a conjugative plasmid and the construction was shown to carry a functioning gene. (It was able to complement *rkpY* mutations.) The DOC-PAGE analysis presented that the polysaccharide pattern of *S. meliloti* 1021 (*rkpY* $^+$) differs from that of the wild-type. While the LMW PS in strain 1021 representing oligoKdo is produced in an abundant amount, the introduction of *rkpY* gene resulted in a significant reduction on this production. Thus, the *rkpY* gene from *S. meliloti* 41 is able to suppress the oligoKdo production in strain 1021.

In order to support our DOC-PAGE results and to prove the exact structures of the polysaccharides that produced by the different mutant strains, analytical examinations were carried out in cooperation with Verena Poinsot (Université Paul Sabatier, Toulouse). These investigations showed that the wild-type *S. meliloti* 41 produces K_R5 antigen and some lipidated Kdo homopolymer (lipo-polyKdo). The *rkpY* mutant bacteria did not produce K_R5 antigen, but synthesized oligoKdo that had no lipid anchor. In case of *rkpY-rkpZ* double mutants, as we supposed no oligoKdo was found. Furthermore, the introduction of *rkpY* gene into *S. meliloti* strain 1021 disturbed Kdo homopolymer synthesis and decreased significantly the amount of oligoKdo. Our conclusions from the genetic experiments and DOC-PAGE analysis were correct but to understand the exact function of the rkpY gene needs a more detailed investigation.

Due to the strain specific *rkp-3* genes and to the *rkpY* that integrated to the genome by horizontal gene transfer, strain 41 of *S. meliloti* became able to produce a special KPS that provided an advantage in symbiotic infection and in defence against bacteriophages. Although,

K_R5 antigen biosynthesis apparatus is the prerequisite of the infection of phage 16-3, the presence of this KPS blocks the invasion of several other rhizobiophages, as we have seen in our experiments. It is interesting to note that bacteriophages provide an important selection force for the horizontal gene transfer of new type of KPS genes and thus bacteriophages may increase the diversification of capsular polysaccharides.

NEW RESULTS

- In contrast to the earlier suggestions, here we demonstrated that the receptor of phage *16-3* is not the K_R5 antigen.
- We managed to identify capsular polysaccharide biosynthesis proteins (RkpM, RkpY, RkpZ) that are essential for the phage infection. Our results suggest that the phage receptor is a protein complex containing RkpM and RkpY subunits. The RkpZ protein may also be related to this complex.
- We have completed the nucleotide sequence of the *rkp-3* region by determining additional DNA sequences around the *rkpY* gene. In the newly sequenced region, we have identified novel putative genes by computational analysis (*orf7343*, *orf8077*, *rkpT2*).
- We have shown in genetic experiments that these putative genes (*orf7343*, *orf8077* and *rkpT2)* play no role in KPS biosynthesis, phage infection and symbiosis.
- We have shown by genetic and biochemical tools, that the LMW polysaccharide produced by the *rkpY* mutant is not a kind of precursor of the K_R5 antigen but a distinct polysaccharide. We have proposed that this polysaccharide is Kdo homopolymer.
- We have shown that the RkpY protein is not only essential for the strain specific K_R5 antigen synthesis, but it also suppresses the Kdo homopolymer production.

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PUBLICATIONS

Publications related to the thesis

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2. **Pálvölgyi, A.**, Deák, V., Poinsot, V., Nagy, T., Nagy, E., Kerepesi, I. and P. Putnoky. (2009): Genetic Analysis of the *rkp-3* Gene Region in *Sinorhizobium meliloti* 41: *rkpY* Directs Capsular Polysaccharide Synthesis to K_R5 Antigen Production. ***Mol. Plant-Microbe Interact.*** 22: 1422-1430 (IF: 4.275).

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2. **Pálvölgyi Adrienn**, Deák Veronika, Hoffmann Gyula, Kerepesi Ildikó, Putnoky Péter: A kapszuláris poliszacharid bioszintézise és a *16-3* fágreceptor, V. Magyar Genetikai Kongresszus Siófok, 2003. (poster)
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6. **Pálvölgyi Adrienn**, Nagy Tibor, Deák Veronika, Nagy Enikő, Kerepesi Ildikó, Putnoky Péter: Az *rkpY* a Kdo-homopolimer bioszintézis szupresszora *Sinorhizobium melilotiban*, Genetikai Műhelyek Magyarországon VII. minikonferencia, MTA Szegedi Biológiai Központ, Szeged, 2008. (oral presentation)

The dissertation can find on: http://www.ttk.pte.hu/biologia/phd/phdfok.htm