***Rhizobium leguminosarum* bv. *trifolii* PssP Protein Is Required for Exopolysaccharide Biosynthesis and Polymerization**

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Rhizobium leguminosarum bv. *trifolii* produces an acidic exopolysaccharide (EPS) that is important for the induction of nitrogen-fixing nodules on clover. Recently, three genes, *pssN*, *pssO*, and *pssP*, possibly involved in EPS biosynthesis and polymerization were identified. The predicted protein product of the *pssP* gene shows a significant sequence similarity to other proteins belonging to the PCP2a family that are involved in the synthesis of high-molecular-weight EPS. An *R. leguminosarum* bv. *trifolii* TA1 mutant with the entire coding region of *pssP* deleted did not produce the EPS. A *pssP* mutant with the 5' end of the gene disrupted produced exclusively low-molecular-weight EPS. A mutant that synthesized a functional N-terminal periplasmic domain but lacked the C-terminal part of PssP produced significantly reduced amounts of EPS with a slightly changed low to high molecular form ratio. Mutants affected in the PssP protein carrying a stable plasmid with a constitutively expressed *gusA* gene induced nodules on red clover that were not fully occupied by bacteria. A mutant with the entire *pssP* gene deleted infected only a few plant cells in the nodule. The *pssP* promoter-*gusA* reporter fusion was active in bacteroids during nodule development.

Additional keywords: exopolysaccharide-deficient mutant, nitrogen fixation.

Bacterial exopolysaccharide (EPS) has been shown to be a critical factor in symbiotic interactions of rhizobia with leguminous plants that form indeterminate type of nodules, for example, *Leucaena*, *Medicago*, *Pisum*, *Trifolium*, and *Vicia* spp. (Becker and Pühler 1998b). Mutants that are defective in EPS production usually induce non-nitrogen-fixing nodules that are not invaded by bacteria, such as nodules induced by *Sinorhizobium meliloti* mutants on alfalfa (Becker and Pühler 1998b; Leigh and Walker 1994), or are only partially infected, such as the ones induced by *Rhizobium leguminosarum* bv. *trifolii* mutants on clover (Bialek et al. 1995; Chakravorty et al. 1982; Chen et al. 1985; Rolfe et al. 1996; Skorupska et al. 1995; van Workum et al. 1997). EPS-deficient mutants of *R. leguminosarum* bv. *viciae* fail to nodulate pea (Borthakur et al. 1986) and induce small, ineffective nodules on vetch (Ivashina et al. 1994; van Workum et al. 1995). The EPS of *R. leguminosarum* is a polymer of octasaccharide subunits, each composed of one galactose (Gal), five glucose (Glc), and two glucuronic acid (GlcA) residues, with acetyl, pyruvyl, and hydroxy-butanoyl

modifications (Fig. 1) (Canter Cremers et al. 1991; Hollingsworth et al. 1988). Similar to *S. meliloti* succinoglycan, high-molecular-weight (HMW) and low-molecular-weight (LMW) fractions of EPS were identified in culture supernatants of *R. leguminosarum* (Djordjevic et al. 1987). LMW polysaccharides are proposed to act as signaling molecules that trigger developmental responses in the host plant or regulate host defense responses. Purified LMW succinoglycan or galactoglucan (EPSII) can partially suppress the nodule invasion defect of EPS-deficient mutants (Gonzales et al. 1996; Wang et al. 1999). The addition of homologous EPS to clover seedlings also restored the nitrogen fixation capability of the *R. leguminosarum* bv. *trifolii* *exo* mutants (Djordjevic et al. 1987).

The polysaccharides are composed of oligosaccharide repeat units that are assembled on a polyisoprenyl-pyrophosphate lipid carrier at the cytoplasmic face of the inner membrane by a sequential transfer of monosaccharides from their nucleotide sugars by the action of specific glycosyltransferases. The oligosaccharides are subsequently translocated, polymerized, and secreted outside the cell (Whitfield et al. 1997). The assembly of the repeating units of EPS of *R. leguminosarum* is initiated by the transfer of glucose 1-phosphate from UDP-glucose to a C₅₅-isoprenylphosphate (IP) carrier (Bossio et al. 1996). The *pssA* gene encoding a glucosyl-IP transferase was identified in *R. leguminosarum* bv. *trifolii* (Janczarek et al. 1999; van Workum et al. 1997) and bv. *viciae* (Borthakur et al. 1988; Ivashina et al. 1994). The *pssC*, *pssD*, and *pssE* genes encoding glycosyltransferases were identified in *R. leguminosarum* bv. *trifolii* (Król et al. 1998; van Workum et al. 1997) and bv. *viciae* (Sadykov et al. 1998). The *pssD* and *pssE* genes encode a glucuronosyl-(β1→4)-glucosyl transferase that catalyzes the second step in biosynthesis of the repeat unit (Pollock et al. 1998). The *pssC* gene product was characterized as the glucuronosyl-(β1→4)-glucuronosyl transferase involved in the third step of repeating subunit assembly (Pollock et al. 1998) (Fig. 1). The *pssG*, *pssH*, and *pssI* genes encoding other glycosyltransferases were also identified (Sadykov et al. 1998). The *exoB* gene encoding a UDP-glucose 4'epimerase was identified in *R. leguminosarum* bv. *trifolii* (Sanchez-Andujar et al. 1997). The *pssB* gene closely linked to *pssA* has been identified as encoding a protein with an inositol monophosphatase activity, and a regulatory function of PssB in the EPS biosynthesis in *R. leguminosarum* bv. *trifolii* was proposed (Janczarek and Skorupska 2001). Previously, the *psi* and *psr* genes, located on the pSym plasmid of *R. leguminosarum* bv. *phaseoli*, engaged in the regulation of EPS biosynthesis have been described (Borthakur and Johnston 1987; Borthakur et al. 1985).

Recently, the sequence of the three closely linked genes *pssN*, *pssO*, and *pssP* of *R. leguminosarum* bv. *trifolii* TA1 has

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been reported (Mazur et al. 2001). The predicted protein product of the *pssP* gene displays a significant structural similarity to members of the membrane-periplasmic auxiliary (MPA1) (Paulsen et al. 1997) or the copolymerase (PCP2a) family (Morona et al. 2000b) that are involved in the synthesis of HMW polysaccharide capsular polysaccharide (CPS) and EPS. They are distinguished on the basis of their sequence similarity, location of transmembrane regions, a characteristic carboxy-terminal ATP-binding domain, and high probability to form coiled-coil regions (Morona et al. 2000b). Recently, several members of the PCP2a family (AmsA of *Erwinia amylovora*, CpsD of *Streptococcus pneumoniae*, ExoP of *S. meliloti*, Wzc of *Escherichia coli*, and Ptk of *Acinetobacter johnsonii*) have been shown to function as autophosphorylating protein tyrosine kinases (Doublet et al. 1999; Ilan et al. 1999; Morona et al. 2000a; Niemeyer and Becker 2001; Vincent et al. 2000). In the cases of Ptk of *A. johnsonii*, CpsD of *Streptococcus pneumoniae*, and ExoP of *S. meliloti*, the ATP-binding motif is required for the autophosphorylating activity of the proteins. The protein tyrosine phosphorylation negatively regulates CPS production in *Streptococcus pneumoniae* (Morona et al. 2000a) and colanic acid synthesis in *E. coli* (Vincent et al. 2000), as well as influences the composition of succinoglycan in *S. meliloti* (Niemeyer and Becker 2001).

The putative PssN protein is highly homologous to the outer membrane auxiliary (OMA) protein family members engaged in the translocation of polysaccharides (Paulsen et al. 1997). PssO amino acid sequence did not reveal any homology with the known bacterial proteins, but it showed characteristic features of the outer membrane proteins, and with PssN and PssP, might constitute a protein complex involved in the polymerization and translocation of EPS across the bacterial membranes (Mazur et al. 2001). Structural similarity of PssP of *R. leguminosarum* bv. *trifolii* and ExoP of *S. meliloti* suggested a functional similarity of these proteins. Becker and colleagues (1995a) have proposed that ExoP regulates the extent of EPS polymerization and thus can influence the ratio of HMW to LMW succinoglycan. There is a suggestion that ExoP regulates the degree of succinoglycan polymerization by controlling the polymerization activities of other proteins, most likely ExoQ and ExoT (Gonzales et al. 1998; Niemeyer and Becker 2001).

To study the possible function of PssP in the EPS biosynthesis of *R. leguminosarum* bv. *trifolii* TA1, we isolated a series of integration mutants disrupted in the *pssP* gene and a mutant with the entire gene deleted. We report here that the deletion of the entire *pssP* gene drastically affected the EPS production and led to a nonmucoid, non-nitrogen-fixing mutant. The *pssP* mutant with a functional N-terminal part but lacking the C-terminal cytoplasmic PssP domain produced a reduced amount of EPS, but the size distribution was only slightly changed. The mutant with the PssP protein disrupted in the periplasmic N-terminal domain produced exclusively an LMW form of EPS. We have also determined that the *pssP* plasmid integration mutants carrying a reporter *gusA* gene on a stable plasmid invaded the nodules, contrary to the mutant with the *pssP* gene deleted. The *pssP* promoter (*PpssP*) cloned in front of a promoterless *lacZ* and *gusA* was functional in free-living cells and in the nodules elicited on red clover by *R. leguminosarum* bv. *trifolii* TA1.

RESULTS

Mutagenesis of the *pssP* gene of *R. leguminosarum* bv. *trifolii* TA1.

Features of the *R. leguminosarum* bv. *trifolii* PssP protein (Mazur et al. 2001) bear similarity to a topological model that has been experimentally established for ExoP of *S. meliloti* and

other proteins belonging to the PCP2a (MPA1-C) family engaged in the synthesis of highly polymerized polysaccharides (Becker et al. 1995a; Morona et al. 2000b; Paulsen et al. 1997). Similar to *S. meliloti* ExoP (Becker et al. 1995a), the PssP protein of *R. leguminosarum* bv. *trifolii* TA1 can be divided into an N-terminal hydrophilic domain (amino acid positions 60 to 449) located probably in the periplasm, with two potential transmembrane helices at the ends that anchor this domain to the inner membrane, and a C-terminal hydrophilic domain (amino acid positions 497 to 746) (Mazur et al. 2001). The 32-amino-acid, proline-rich motif conserved in the ExoP family (RX₄AX₂RX₄APKX₁₁GX₃G) was found to partially overlap the second transmembrane helix (amino acid positions 433 to 464). The hydrophilic, probably cytoplasmic, C-terminal domain of PssP contains the conserved Walker motif A (SSLPGEGKS) (Walker et al. 1982) for ATP-binding, amino acid positions 561 to 569 (Mazur et al. 2001) and the Walker motif B (DYIIVDLPP) at amino acid positions 668 to 676. In the N-terminal domain, four regions (amino acid positions 205 to 234, 259 to 290, 354 to 381, and 394 to 424) with a very high probability ($P \approx 1.0$, 28-amino acid [aa] window) to form coiled-coils were identified. Coiled-coil regions, both in periplasmic and cytoplasmic carboxy-terminal domains of PCP2a proteins, were found (Morona et al. 2000b) that could be involved in interactions with other components of CPS/EPS synthesis or export machinery (Morona et al. 2000b). Besides a significant amino acid sequence identity (38.8%) and structural similarity between PssP and ExoP, we observed an essential difference between both proteins, namely, the lack of the tyrosine-rich region at the carboxy-terminal part of PssP that could influence the function of the protein.

To investigate the function of the *pssP* gene in EPS production, three genomic mutants were generated by gene replacement or plasmid integration strategies (Fig. 2). The *pssP* deletion mutant RtP22Δ was constructed with a suicide plasmid pKPA carrying a Gm^r cassette in place of the entire *pssP* gene. The construct with a deletion comprising the complete *pssP* coding region (starting at 36 base pairs [bp] upstream of the promoter region to 385 bp downstream of the *pssP* gene) was transferred to wild-type strain RtTA1, resulting in replacement

Fig. 1. Structure of the repeating unit of exopolysaccharide of *Rhizobium leguminosarum* bv. *trifolii* strain LPR5 (Pollock et al. 1998; van Workum et al. 1997). Glc = glucose, Gal = galactose, GlcA = glucuronic acid, Pyr = pyruvate, and Ac = acetate.

of the wild-type *pssP* gene by the Gm^r cassette in the homogenote RtP22Δ (Fig. 2C). This mutant was completely deficient in EPS production, and no detectable material could be obtained either by ethanol precipitation (Table 1) or by lyophilization of cell-free culture supernatant.

The *pssP* mutant designated RtP20 was constructed by integration of plasmid pH51 carrying the intergenic region *pssO-pssP* and the 5' end of *pssP* encoding the N-terminal part of the PssP protein with a Km^r cassette in the *Bam*HI site (Fig. 2C). Integration of the hybrid plasmid into the RtTA1 genome by a single crossover resulted in disruption of the *pssP* coding region, and the merodiploid strain RtP20 was created (Fig. 2C). This mutant harboring the functional N-terminal periplasmic domain of PssP (1 to 478 aa) showed a significant decrease in EPS production in 79CA medium (Table 1).

The next *pssP* mutant, designated RtP23, was created by integration of plasmid pKP23Gm that carried the intergenic region *pssO-pssP* and the 5' end of *pssP* encoding the N-terminal part of PssP with an Ω-Gm cassette in the *Bam*HI site of the

coding region (Fig. 2C). Plasmid pKP23Gm was transferred to wild-type strain RtTA1, giving rise to the merodiploid strain RtP23. Disruption of the *pssP* gene was further analyzed by Southern hybridizations and polymerase chain reaction (PCR) analyses, which showed that the 5' end of *pssP* was indeed interrupted by Ω-Gm, with a plasmid vector integrated upstream of the *pssP* gene (Fig. 2C). Because of the disruption, only a short N-terminal PssP domain (1 to 253 aa) was active. The RtP23 mutant also synthesized a reduced amount of EPS on 79CA medium in comparison to wild-type strain RtTA1 (Table 1).

EPS production in *pssP* mutants.

Mutant RtP22Δ with the entire *pssP* coding region deleted did not produce any detectable amount of EPS, which indicated that the PssP protein was indispensable for EPS production. The RtP20 and RtP23 mutants, characterized as partial merodiploids disrupted in the *pssP* gene, produced a reduced amount of EPS in comparison to wild-type strain RtTA1 (Table 1).

Fig. 2. A, Physical and genetic maps of cosmid pARF136 carrying the *pssN*, *pssO*, and *pssP* genes of *Rhizobium leguminosarum* bv. *trifolii* TA1 encoding the putative proteins involved in exopolysaccharide polymerization and export. B, Constructs containing a putative *pssP* promoter cloned upstream of the promoterless *lacZ* gene into vector pMP220. The predicted promoter sequences upstream of the *pssP* gene are shown by solid rectangles. C, Constructs of *R. leguminosarum* bv. *trifolii* *pssP* mutants used for integration and replacement mutagenesis are shown on the left. On the right, the resulting genomic structures of RtP20, RtP23, and RtP22Δ mutants are indicated. The inactive parts of the *pssP* gene are in parentheses. The heavy lines indicate the vector part of the integrated plasmid. B = *Bam*HI, Bg = *Bgl*II, E = *Eco*RI, K* = *Kpn*I, P = *Pst*I, S = *Sal*I, and X* = *Xba*I. Restriction sites derived from multiple cloning sites of the vector due to intermediate cloning steps are indicated by asterisks.

EPS isolated from culture supernatants of wild-type strain RtTA1 and mutants RtP20 and RtP23 were fractionated by Bio-Gel A5m gel filtration chromatography (Bio-Rad, Hercules, CA, U.S.A.) and the ratios of two EPS fractions that significantly differed in molecular weights were calculated (Fig. 3; Table 1). An insignificant change in the HMW to LMW EPS ratio, in favor of LMW, was observed in the RtP20 mutant, producing a functional N-terminal but lacking the C-terminal PssP domain when compared with the EPS isolated from wild-type strain RtTA1 (Fig. 3B; Table 1). EPS isolated from the RtP23 mutant with a disrupted N-terminal PssP domain produced almost exclusively an LMW form of EPS (Fig. 3C). In conclusion, the phenotypes of these two mutants indicated the importance of the functional N-terminal domain in the extent of EPS polymerization, i.e., in determining the ratio of HMW to LMW EPS.

To confirm that both the HMW and LMW fractions represented EPS, peak samples from Bio-Gel A5m chromatography (Fig. 3) were analyzed for monosaccharide composition. The glucose/glucuronic acid/galactose ratio was found to be approximately 5:2:1, characteristic for the acidic EPS of *R. leguminosarum*.

Symbiotic phenotype of *pssP* mutants.

The wild-type strain RtTA1 mutated in the *pssP* gene induced nodules on red clover (Table 2). The nonmucoid RtP22Δ mutant induced white nodules with a 7-day delay when compared with wild-type strain RtTA1. Similar to other nonmucoid mutants of *R. leguminosarum* bv. *trifolii* (Skorupska et al. 1995), the RtP22Δ mutant was not able to fix nitrogen (Table 2). Plasmid integration mutants RtP20 and RtP23 induced pink, elongated nodules on *T. pratense* without delay and the plants appeared green, indicating nitrogen fixation (Table 2). Mutant RtP23 induced a significantly decreased number of nodules on red clover. We did not observe any apparent effects of *pssP* disruption in these merodiploid strains on the level of nitrogenase activity (data not shown).

With the use of a stable plasmid pJBA21Tc harboring a constitutively expressed *gusA* gene (Wielbo and Skorupska 2001), the invasion behavior of the wild-type and mutant strains was studied (Fig. 4A to D). The plasmid pJBA21Tc was introduced by triparental conjugation into RtTA1, RtP22Δ, RtP20, and RtP23, and the nodules elicited by exconjugants were examined for GUS activity. Only a few plant cells were invaded by the RtP22Δ mutant in the 4-week-old nodules, in contrast to the fully infected nodules elicited by wild-type strain RtTA1 (Fig. 4A and D). Mutants RtP20 and RtP23 invaded the nodule plant cells, but GUS activity was more pronounced in the younger part of nodules exhibiting a premature senescence of the bacteroids (Fig. 4B and C). For these mutants, GUS activity was observed in the infection and nitrogen-fixing zones of nodules, but the extent of nodule occupation was diminished when compared with RtTA1-elicited nodules. The disappearance of GUS activity could indicate a loss of bacterial viability caused by a defense response directed toward bacteroids in the senescence zone.

Transcriptional activity of *PpssP*.

Computer searches identified the presence of two putative promoters upstream of the *pssP* gene located –86 to –41 and –13 to +32 bp from the putative transcriptional start site (Fig. 2B). The first putative promoter sequence partially overlapped the 3' end of the *pssO* gene. To investigate the transcriptional activity of *PpssP*, the region upstream of *pssP* was cloned in front of a promoterless *lacZ* gene into a mobilizable vector pMP220. Inserts in the pMP2P and pMP3P plasmids essentially differed only in the 5' end and overlapped either both pu-

tative promoters of *pssP* (the pMP2P construct) or one of them (the pMP3P construct) (Fig. 2B). The *PpssP-lacZ* gene fusions were introduced into *E. coli* DH5α and wild-type strain RtTA1, and the level of β-galactosidase activity was measured (Table 3). Both constructs were active in *E. coli* DH5α and *R. leguminosarum* bv. *trifolii* genetic backgrounds, but the transcription level was very low in *E. coli*. In this strain, a twofold-higher activity was observed with pMP3P, harboring the shorter insert. Contrary to that, in the RtTA1 background, the level of β-galactosidase expression was higher with the longer construct pMP2P, indicating the importance of upstream sequences in the initiation of transcription. In the presence of clover root exudate as a coinducer, an about 1.55- to 2-fold induction of the *PpssP* activity was observed with pMP2P and pMP3P plasmids (Table 3), indicating a possibility of symbiotic regulation of the *pssP* gene.

To investigate the transcriptional activity of *PpssP* in nodules, a stable reporter plasmid was constructed containing the *PpssP* region fused to the promoterless *gusA* gene. The obtained pPT12 plasmid was introduced into wild-type strain RtTA1 and the selected Tc^r exconjugants were used for inoculation of clover seedlings. Nodules were harvested from 10 days up to 3- and 5-weeks after inoculation, hand-sectioned, and stained for the presence of GUS activity. Expression of the *PpssP-gusA* fusion was observed throughout the nodules examined at different stages of development, as shown in Figure 4E to G. These observations also indicated that the constructed reporter plasmid might be useful in monitoring the transcriptional activity of rhizobial promoters in developing and mature nodules.

DISCUSSION

Recently, we identified three new genes in an *R. leguminosarum* bv. *trifolii* TA1 pARF136 cosmid insert, *pssP*, *pssO*, and *pssN* (Mazur et al. 2001). On the basis of computer analysis and sequence similarity to the known proteins, the deduced gene products PssP and PssN have been proposed to function in the polymerization and export of EPS. The PssP and PssN proteins may be a part of the type 1 secretion system found in gram-negative bacteria that employs the cytoplasmic-MPA1 and OMA proteins to export EPS (Paulsen et al. 1997). Additionally, we have identified a putative PssO gene product that has no homologues among any known bacterial proteins but whose hydropathy plot indicates outer membrane localization. We speculate that this protein might be a constituent of the type 1 system involved in the polymerization and export of EPS in *R. leguminosarum* bv. *trifolii* TA1.

The PssP protein could be included in the PCP2a or MPA1 family that consists of both gram-negative and gram-positive

Table 1. Exopolysaccharide (EPS) production by *Rhizobium leguminosarum* bv. *trifolii* TA1 and the *pssP* mutants

Strain	Total EPS ^a mg liter ⁻¹ (% of control)	HMW/LMW EPS ^b (%)
RtTA1	1,635.7 (100)	49.8:50.2
RtP20	696.6 (42.8)	42:58
RtP23	1,133.6 (69.7)	15:85
RtP22Δ	0	0

^a Amount of ethanol-precipitable EPS from the culture growing on 79CA medium supplemented with 0.5% glycerol is expressed as glucose equivalents (Loewus 1952).

^b High-molecular-weight/low-molecular-weight (HMW/LMW) proportions were calculated as ratio of peak areas of EPS chromatographed on a Bio-Gel A5m column (Bio-Rad, Hercules, CA, U.S.A.). Values are means of four independent measurements. Standard deviations were equal to or less than 10%.

bacterial members involved in the synthesis of HMW polysaccharides (CPS and EPS) (Becker et al. 1995a; Morona et al. 2000b; Paulsen et al. 1997). PssP amino acid sequence and its secondary structure resemble ExoP from *S. meliloti*, which functions in the polymerization and export of succinoglycan. Recently, similar to other members of the PCP2a family, the autophosphorylating protein tyrosine kinase activity of ExoP was shown (Niemeyer and Becker 2001). Periplasmic localization of a 481-residue loop and cytoplasmic localization of an

about 280-aa C-terminal domain of *S. meliloti* ExoP have been supported experimentally (Becker et al. 1995a). The proline-rich motif in the N-terminal domain and the Walker A ATP-binding motif at the carboxy-terminal domain are involved in ExoP activity (Becker and Pühler 1998a; Niemeyer and Becker 2001). Some substitutions within the proline-rich motif resulted in enhanced production of LMW EPSI at the expense of HMW EPSI in *S. meliloti* mutants (Becker and Pühler 1998a). Mutations in the Walker A ATP-binding motif prevented the ATPase activity and phosphorylation of tyrosine residues of ExoP. The ATP-binding motif mutants synthesized only the octasaccharide subunit of succinoglycan and were completely unable to polymerize it (Niemeyer and Becker 2001). Also, Gonzales and colleagues (1998) showed that the *S. meliloti* *exoP* mutant with a Tn5 insertion within the N-terminal periplasmic domain blocked any polymerization of the succinoglycan octasaccharide subunits. On the basis of these results, the authors suggested not a regulatory but rather a critical role of ExoP in succinoglycan biosynthesis, e.g., enzymatic, by catalyzing the formation of dimers of the octasaccharide subunits, or structural, by forming a complex with ExoQ and ExoT proteins, necessary for their function (Gonzales et al. 1998). The *exoQ* and *exoT* genes participate in the synthesis or secretion of succinoglycan. ExoQ is required for the production of HMW succinoglycan, and ExoT is involved in the synthesis or secretion of the LMW dimers and trimers but not the monomers (Gonzales et al. 1998).

In our study, the RtP22Δ mutant with the entire *pssP* gene deleted was completely deficient in EPS synthesis, which indicated the importance of this gene in EPS production in *R. leguminosarum* bv. *trifolii* TA1. The RtP20 mutant with an active N-terminal domain of PssP and an inactive carboxy-terminal part of the protein produced a significantly reduced amount of EPS (42.8%) that was not essentially altered in the ratio of HMW to LMW in comparison to wild-type strain RtTA1 (Fig. 3; Table 1). On the basis of mutant phenotype, it was concluded that the C-terminal part of the PssP protein influences the total level of EPS produced. Mutant RtP23, in which only a short N-terminal part of PssP was active, exhibited a severe defect in the production of HMW EPS (Fig. 3C). The total extracellular polysaccharide produced by this mutant was made up of LMW forms, indicating the importance of the N-terminal domain of PssP in the EPS subunit polymerization.

Both mutants, RtP20 and RtP23, were able to fix nitrogen but differed in the extent of nodule occupation and bacteroid viability when compared with wild-type strain RtTA1. These data suggested that the LMW EPS may act as a biologically active signal in the symbiotic interaction of *R. leguminosarum* bv. *trifolii* with clover. In *S. meliloti* strain 1021, a trimer fraction of succinoglycan was proposed to be the symbiotic signal during host plant infection (Gonzales et al. 1998; Wang et al.

Fig. 3. Gel filtration chromatography of exopolysaccharides (EPS) produced by **A**, *Rhizobium leguminosarum* bv. *trifolii* wild-type strain RtTA1 and the plasmid integration mutants **B**, RtP20 and **C**, RtP23. EPS was fractionated on a Bio-Gel A5m column (Bio-Rad, Hercules, CA, U.S.A.) as described in text. The retention times of dextran blue (2 MDa), dextran T250 (250 kDa), and dextran T10 (10 kDa) molecular mass markers are indicated by arrows.

Table 2. Symbiotic properties of the *pssP* mutants of *Rhizobium leguminosarum* bv. *trifolii* TA1^a

Mutant strain	Nodules per plant (no.)	Green wet wt per plant (mg)	Symbiotic phenotype
RtTA1 (wild type)	22.6 ± 3.3	68.6 ± 8.8	Nod ⁺ Fix ⁺
RtP20	18.6 ± 2.4	56.9 ± 4.4	Nod ⁺ Fix ⁺
RtP23	15.4 ± 1.4 ^b	63.2 ± 10.6	Nod ⁺ Fix ⁺
RtP22Δ	10.3 ± 4.5 ^b	20.7 ± 5.6 ^b	Nod ⁺ Fix ⁻
Uninoculated control	0	22.1 ± 4.4 ^b	NA ^c

^a Symbiotic properties of rhizobia were measured 28 days after inoculation. The given values (± standard deviation) are averages of at least four independent experiments with 20 plants for each treatment.

^b Mean values differ significantly ($P = 0.05$) from control values according to Student's *t* test.

^c NA = not applicable.

Fig. 4. Sections of nodules formed by **A**, wild-type strain RtTA1; plasmid integration mutants **B**, RtP20 and **C**, RtP23; and **D**, mutant RtP22 Δ with the entire *pssP* gene deleted. These strains carried the stable plasmid pJBA21Tc with a constitutively expressed *gusA* gene. Nodules were harvested 28 days after clover inoculation, hand-sectioned, and stained with 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (X-gluc) for GUS activity. **E to G**, Sections of nodules elicited by wild-type strain RtTA1 carrying plasmid pPT1*par* carrying the *pssP* gene promoter cloned in front of a promoterless *gusA*-Sp^r cassette (Sp = spectinomycin). Nodules were harvested **E**, 10; **F**, 21; and **G**, 35 days after inoculation, hand-sectioned, and stained for GUS activity.

1999). Addition of succinoglycan trimer in trans rescued the nodule invasion defect of the *exoY* mutant (Wang et al. 1999). Alfalfa nodule invasion can be also mediated by other symbiotically important polysaccharides, such as EPSII or K antigen produced by *S. meliloti* strains under specific environmental conditions (Gonzales et al. 1996; Pellock et al. 2000; Reuhs et al. 1995).

Promoter of the *pssP* gene was active both in free-living cells and in nodules elicited by wild-type strain RtTA1. A substantially elevated level of transcription, additionally enhanced in the presence of root exudates, was observed when the construct with a long 5' sequence preceding the *pssP* gene was used (Table 3). This indicated the possibility of formation of a protein complex specific for *Rhizobium* spp. that influenced the level of transcription. Enhanced expression of the *exoP* gene was also observed in *S. meliloti* in the presence of luteolin (Dusha et al. 1999). The presence of respective flavonoids in the growth media increased the production of EPS in *S. meliloti* (Dusha et al. 1999) and in *R. fredii* (Dunn et al. 1992). There is a possibility that the flavonoids directly affect the EPS synthesis by accumulating in the inner membrane and changing the conformation of membrane-localized enzymes involved in the biosynthesis and polymerization of EPS (Recourt et al. 1989). This also suggests a possibility of coregulation of Nod factors and EPS biosynthesis (Dusha et al. 1999).

When clover plants were infected with wild-type strain RtTA1 harboring the *PpssP-gusA* fusion, we observed GUS activity in young and mature nodules, indicating the activity of *PpssP* during all stages of the symbiosis. Such may suggest that, in clover nodules, some level of EPS synthesis is necessary in both the early and late stages of nodulation. There are notions that at least some *exo* genes are down-regulated in planta (Latchford et al. 1991; Reuber et al. 1991). *TnphoA* fusions with *exoF*, *exoP*, and *exoA* genes were active only in the early symbiotic zone of the nodule (Reuber et al. 1991). This suggested that EPSI synthesis might not be necessary in later stages of nodulation. Latchford and colleagues (1991) found that, in *Phaseolus* spp., nodules of the *pssA-gusA* fusion was not expressed in bacteroids of *R. leguminosarum* bv. *phaseoli*, whereas this fusion was transcribed in free-living cells. The *psi* gene product of *R. leguminosarum* (Borthakur et al. 1985) and the *exoR* gene product of *S. meliloti* (Doherty et al. 1988; Reuber et al. 1991) were described as negative regulators of EPS biosynthesis in free-living cells and were thought to be involved in repressing EPS production in the nodule. Our results indicate that transcription of the *pssP-gusA* gene fusion was not repressed by the *psi* or other gene products in clover nodules. Further experiments are needed to explain the regulation of EPS biosynthesis in nodules.

Table 3. The *pssP* promoter activity measured by the level of β -galactosidase in *Escherichia coli* DH5 α and *Rhizobium leguminosarum* bv. *trifolii* TA1 backgrounds

Plasmids	β -galactosidase activity ^a in:			Fold
	<i>E. coli</i>	<i>R. leguminosarum</i> bv. <i>trifolii</i> TA1		induction in RfTA1
	DH5 α	– Root exudate	+ Root exudate ^b	
pMP2P	7.92	273.82	588.85	2.15
pMP3P	14.63	164.78	255.52	1.55
pMP220 vector	0.92	85.52	91.08	1.06
None (background)	0.12	53.65	49.42	0.92

^a β -Galactosidase activity values are given in Miller units.

^b Clover root exudate (10 μ mol/liter) prepared according to Zaai and colleagues (1988) was used as a coinducer. Values are means of at least four independent experiments with a standard error less than 15%.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media.

Bacterial strains and plasmids used in this work are listed in Table 4. *R. leguminosarum* strains were grown at 28°C in 79CA complete medium (Vincent 1970) with 1% mannitol or 0.5% glycerol as carbon sources. *E. coli* strains were grown at 37°C in Luria-Bertani medium (Sambrook et al. 1989). Antibiotics for *E. coli* and *R. leguminosarum* cultures were added to the following final concentrations: tetracycline (Tc), 10 μ g ml⁻¹; rifampicin (Rif), 40 μ g ml⁻¹; kanamycin (Km), 30 to 40 μ g ml⁻¹; gentamicin (Gm), 5 to 10 μ g ml⁻¹; spectinomycin (Sp), 40 μ g ml⁻¹; and ampicillin (Ap), 100 μ g ml⁻¹.

DNA manipulation.

Standard techniques were used for plasmid isolation, restriction enzyme digestion, agarose gel electrophoresis, cloning, labeling of DNA, and Southern hybridization (Sambrook et al. 1989). DNA probes for Southern hybridization were labeled with nonradioactive digoxigenin DNA Labeling and Detection Kit (Boehringer Mannheim, Mannheim, Germany). Nucleotide sequence of the 6.0-kb fragment carrying the *pssN*, *pssO*, and *pssP* genes is available in the EMBL database under the accession number AF067140. The putative promoter sequences were predicted by using Neural Network Promoter prediction at BDGP (Berkeley Drosophila Genome Project, Berkeley, CA, U.S.A.).

Mutagenesis of the *pssP* gene.

Construction of the RtP20 and RtP23 integration mutants. Integration mutagenesis of the *pssP* gene was accomplished by the use of pBUC1 and pK18/19mobGII suicide integrative vectors. DNA fragments used for plasmid integration mutagenesis were subcloned in several steps into mobilizable vectors by using appropriate restriction sites. First, plasmid pH5 was constructed by subcloning the 1.45-kb *Bgl*III-*Eco*RI fragment of cosmid pARF136 carrying the intergenic region *pssO-pssP* and the 5' end of the *pssP* gene into the pBUC1 vector. The 3-kb Ω -Km cassette derived from the pSUP2021 plasmid was then inserted into the *Bam*HI site of the *pssP* coding region, which resulted in plasmid pH51. The orientation of the Ω -Km cassette in the construct was confirmed by restriction analysis.

The hybrid plasmid pKP23Gm was constructed by subcloning the 1.45-kb *Xba*I*-*Eco*RI (restriction sites derived from multiple cloning sites of the vector because of intermediate cloning steps are indicated by asterisks) fragment of pH5 carrying the intergenic region *pssO-pssP* and the 5' end of *pssP* into the pK18mobGII vector. The Ω -Km cassette derived from pMS272 (Becker et al. 1995b) was then cloned into the *Bam*HI site of the *pssP* coding region, resulting in pKP23Gm. Thus constructed, plasmids pH51 and pKP23Gm were transferred from *E. coli* S17-1 (Simon et al. 1983) to *R. leguminosarum* bv. *trifolii* TA1. Integration of hybrid plasmids into the *R. leguminosarum* bv. *trifolii* genome by single-cross-over events was selected for by the vector-encoded antibiotic resistance. The *Rhizobium* mutant obtained by the integration of pH51 was designated RtP20. In this mutant, the *lac* vector promoter is oriented in the direction of transcription, but the promoter of Ω -Km is in an opposite orientation to the 3' part of the *pssP* gene. Thus, we expected no functional C part of the PssP protein. In the mutant RtP23, obtained by integration of pKP23Gm, the *lac* vector promoter is in an opposite orientation to the transcription of the entire *pssP* gene (Fig. 2C). Recombination between the hybrid plasmids and the *pssP* gene of the *R. leguminosarum* bv. *trifolii* TA1 genome was verified by Southern hybridizations by using the total DNA of mutants digested with different restriction enzymes and several probes,

as described below. Genomic recombination in the RtP20 mutant was tested by using the 1.45-kb *Bgl*II-*Eco*RI fragment of pARF136 and the 3-kb *Bam*HI fragment carrying Ω -Km as probes. The RtP23 mutant was tested by using the 1-kb *Bam*HI fragment carrying Ω -Gm, the 1.45-kb *Bgl*II-*Eco*RI fragment of pARF136, and the 2.6-kb *Bam*HI-*Bgl*III fragment partially overlapping the *pssP* gene as probes.

Construction of the RtP22 Δ mutant. To create a deletion of *pssP* by marker replacement, a pKPA plasmid was constructed in which the gene encoding gentamicin resistance was flanked by the upstream and downstream DNA regions of the *pssP* gene. First, the 651-bp *Eco*RI-*Pst*I* fragment carrying the *pssO* gene adjacent to the 5' end of *pssP* from cosmid pARF136 was cloned into the pK19mobGII vector. The 1-kb *Pst*I-*Sph*I Ω -Gm cassette obtained by PCR amplification of the gene responsible for gentamicin resistance from pBBR1MCS-5 (Kovach et al. 1995) was then added to the construct. In the next step, the 648-kb *Sph*I-*Hind*III* fragment from cosmid pARF136 neighboring with the 3' end of *pssP* was subcloned into the construct, resulting in the pKPA plasmid. This plasmid was transferred from *E. coli* S17-1 to *R. leguminosarum* bv. *trifolii* TA1 and a double-crossover event was induced by selecting for the cassette-encoded gentamicin resistance. This resulted in strain RtP22 Δ . The recombination event was verified by Southern hybridization by using the 2.6-kb *Bam*HI-*Bgl*III fragment of pARF136 partially overlapping the *pssP* gene and the 1.45-kb *Bgl*II-*Eco*RI fragment of pARF136, also partially overlapping *pssP*, as probes.

Construction of plasmids with a putative PpssP. By using appropriate restriction sites, the DNA fragments containing the putative PpssP were cloned in front of a promoterless *lacZ* gene

into the pMP220 vector (Spaink et al. 1987). Plasmid pMP2P contained the longer, 1.1-kb *Bam*HI-*Pst*I fragment (–429 to +671 bp) and plasmid pMP3P contained the shorter, 707-bp *Bgl*II-*Pst*I fragment (–36 to +671 bp). The resulting hybrid plasmids were transferred by triparental mating into *R. leguminosarum* bv. *trifolii* TA1.

To study the expression of the PpssP in planta, the stable plasmid named pPT1par was constructed. The 1.1-kb *Bam*HI-*Pst*I fragment (–429 to +671 bp) containing the putative PpssP was cloned into vector pRK7813 in an opposite orientation to the *lac* vector promoter. Immediately downstream of the putative PpssP, the promoterless *gusA*-Sp^r cassette from mTn5gusA40 (Wilson et al. 1995) was inserted into the *Eco*RI-*Hind*III site of pRK7813. The *Hind*III fragment containing the *parDE*-Gm^r genes (Becker et al. 1995b; Weinstein et al. 1992) was then incorporated into the construct, giving rise to the pPT1par plasmid. This plasmid was transferred by triparental mating into *R. leguminosarum* bv. *trifolii* TA1, and transconjugants were selected on 79CA medium with tetracycline (10 μ g ml^{–1}) and X-Glc (50 μ g ml^{–1}).

A stable plasmid vector pJBA21Tc carrying a constitutively expressed *gusA* gene for studying nodule invasion was described earlier (Wielbo and Skorupska 2001). This plasmid was transferred to wild-type strain RtTA1 and the *pssP* mutants RtP20, RtP23, and RtP22 by triparental mating, followed by selection for tetracycline resistance and the ability to express *gusA*.

β -Galactosidase assay.

R. leguminosarum bv. *trifolii* cells were grown to an optical density at 600 nm of 0.6 to 0.8 and the levels of β -galactosi-

Table 4. Bacterial strains and plasmids used in this study

Strain	Relevant characteristics ^a	Reference
<i>Rhizobium leguminosarum</i> bv. <i>trifolii</i>		
TA1	Wild type, Str ^r , Rif ^r	Chakravorty et al. 1982
RtP20	TA1 with <i>pssP</i> interrupted by integration of plasmid pH51, Gm ^r , Km ^r	This work
RtP23	TA1 with <i>pssP</i> interrupted by integration of pKP23Gm, Gm ^r , Km ^r	This work
RtP22 Δ	TA1 with the entire <i>pssP</i> gene deleted, Gm ^r	This work
<i>Escherichia coli</i>		
DH5 α	<i>supE44</i> Δ <i>lacU169</i> (ϕ 80 <i>lacZ</i> Δ M15) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>	Sambrook et al. 1989
S17-1	294 derivative RP4-2-Tc::Mu-Km::Tn7 chromosomally integrated	Simon et al. 1983
Plasmids		
pBBR1MCS-5	<i>mob</i> , Gm ^r cloning vector	Kovach et al. 1995
pK18/19mobGII	pUC18/19 derivatives, <i>lacZ</i> α , <i>mob</i> , <i>gusA</i>	Katzen et al. 1999
pMP220	IncP, <i>mob</i> , promoterless <i>lacZ</i> , Tc ^r	Spaink et al. 1987
pMS272	Promoterless <i>lacZ</i> -Gm interposon	Becker et al. 1995b
pRK7813	IncP, <i>mob</i> , Tra [–] cosmid vector, Tc ^r	Stanley et al. 1987
pTR101	RK2 <i>par</i> derivative	Weinstein et al. 1992
pSUP2021	pBR325 derivative, Cm ^r , Km ^r , Ap ^r , <i>mob</i>	Simon et al. 1983
pARF136	pLAFR3 with 19-kb insert of TA1 carrying <i>pss</i> genes	Król et al. 1998
pJBA21Tc	pJB321 derivative carrying constitutively expressed <i>gusA</i> and Tc ^r cassette	Wielbo and Skorupska 2001
pBUC1	pUC19 and pBBR1 MCS-5 derivative: <i>oriC</i> , <i>mob</i> , MCS-5, Ap ^r , Gm ^r	This work
pH5	pBUC1 with 1.45-kb <i>Bgl</i> II- <i>Eco</i> RI fragment of pARF136 carrying 5' end of <i>pssP</i>	This work
pH51	pH5 derivative carrying 1.45-kb <i>Bgl</i> II- <i>Eco</i> RI fragment of pARF136 with 3-kb Ω -Km in <i>Bam</i> HI site of <i>pssP</i>	This work
pKPA	pK19mobGII with <i>Eco</i> RI- <i>Hind</i> III* fragment of pARF136 with Gm ^r cassette in place of the entire <i>pssP</i>	This work
pKP23Gm	pK18mobGII derivative carrying 1.45-kb <i>Xba</i> I*- <i>Eco</i> RI fragment of pH5 with 1-kb Ω -Gm in <i>Bam</i> HI site of <i>pssP</i>	This work
pMP2P	pMP220 with 1.1-kb <i>Bam</i> HI- <i>Pst</i> I fragment of pARF136 carrying the <i>pssP</i> promoter	This work
pMP3P	pMP220 with 707-bp <i>Bgl</i> II- <i>Pst</i> I pARF136 carrying the <i>pssP</i> promoter	This work
pPT1par	pRK7813 carrying promoter of <i>pssP</i> , promoterless <i>gusA</i> -Sp ^r cassette, and <i>parDE</i> -Gm ^r genes from pTR101 and pMS272	This work

^a Ap = ampicillin, Cm = chloramphenicol, Gm = gentamicin, kb = kilobases, Km = kanamycin, IncP = P group of plasmid incompatibility, *mob* = mobility site, Rif = rifampicin, Sp = spectinomycin, Str = streptomycin, Tc = tetracycline, and Tra[–] = defect in transfer. Restriction sites derived from multiple cloning sites of the vector because of intermediate cloning steps are indicated by asterisks.

dase were determined according to Rosas and colleagues (1998). β -Galactosidase activity values are given in Miller units.

GUS histochemistry.

For histochemical analysis, the handmade sections of clover root nodules were stained in 50 μ g of 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (X-gluc) solution per ml in 50 mM sodium phosphate buffer (pH 7.2) with 0.1 mM EDTA, 0.38 mM $K_3Fe(CN)_6$, and 0.38 mM $K_4Fe(CN)_6$ for about 1 h at room temperature (Wilson et al. 1995). Stained nodule sections were observed with a Nikon light microscope (OPTIPHOT2; Nikon, Tokyo, Japan) using Nomarsky optics.

EPS analysis.

For the EPS isolation, 120-ml cultures of TA1, RtP20, and RtP23 strains were grown in 79CA medium with 0.5% glycerol for 5 days at 28°C in a rotary shaker. After a centrifugation step, the cell-free supernatant was subjected to protein coagulation by heating for 5 min in boiling water. The denaturated proteins were pelleted by centrifugation (14,000 $\times$ g at 4°C for 30 min) and the supernatant was desalted by Sephadex G10 chromatography (Becker et al. 1998). Finally, the EPS solution was lyophilized. The dried EPS was dissolved in 0.1 M sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl to a final concentration of 5 mg/ml and separated by column chromatography on Bio-Gel A5m. Two milliliters of this solution was loaded onto the column and 2-ml fractions were collected. Fractions were assayed for carbohydrates according to Loewus (1952), and total sugar content was calculated in glucose equivalents. For EPS quantification in culture supernatants, EPS was precipitated with 10 volumes of ethanol, lyophilized, resolved in water, and analyzed for carbohydrate content.

Monosaccharide composition of the LMW and HMW EPS fractions was determined by combined gas chromatography/mass spectrometry analyses of their corresponding alditol acetate derivatives obtained following methanolysis, carboxyl reduction, and hydrolysis as previously described (Russa et al. 1996).

Plant tests.

R. leguminosarum bv. *trifolii* strains were assayed for their symbiotic phenotypes on *Trifolium pratense* (cv. Ulka). Seeds were surface-sterilized, germinated, and grown as described previously (Bialek et al. 1995). The clover plants were inspected for root nodule formation after 4 to 5 weeks. Nitrogen fixation was evaluated indirectly, by designating the plants Fix^+ and Fix^- on the basis of color and green matter production, which was estimated by weighing the shoots.

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