Research Note

Molecular Characterization of *pssCDE* Genes of *Rhizobium leguminosarum* bv. *trifolii* strain TA1: *pssD* Mutant Is Affected in Exopolysaccharide Synthesis and Endocytosis of Bacteria

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We have identified the three genes *pssCDE* in *Rhizobium leguminosarum* bv. *trifolii* TA1. Even though they were almost identical to earlier identified *pssCDE* genes of *R. leguminosarum*, they differed in gene lengths and gene overlaps. The predicted gene products of *pssCDE* genes shared significant homology to prokaryotic glycosyl transferases involved in exopolysaccharide synthesis. The Tn5 insertion in *pssD* created the nonmucoid mutant that induced non-nitrogen-fixing nodules. The microscopic analysis of the nodules, induced on *Trifolium pratense* by the *pssD133* mutant, showed abnormally enlarged infection threads densely packed with bacteria, which were released from the infection threads in an unusual way. The symbiosomes were observed very rarely and the nodule remained almost empty. Symbiotic phenotype of the *pssD133* suggested a correlation between this mutation and defective endocytosis of bacteria into nodule cells.

Acidic exopolysaccharide (EPS) synthesized by rhizobia is important for the induction of nitrogen-fixing nodules on leguminous plants such as alfalfa, clover, or pea, which form indeterminate type of nodules (reviewed in Diebold and Noel 1989; Leigh and Walker 1994; Noel 1992). Mutants of *R. leguminosarum* bvs. *trifolii* and *viciae* deficient in EPS synthesis (*Exo*[−]) formed nodules that were partially infected, but did not fix nitrogen (Chen et al. 1985; Derylo et al. 1986; Rolfe et al. 1996; Skorupska et al. 1995; van Workum et al. 1995, 1997). Nodules induced on clover by *Exo*[−] mutants of *R. leguminosarum* bv. *trifolii* were poorly developed, but infection threads occurred and bacteria were released into plant cells (Bialek et al. 1995; Skorupska et al. 1995; Rolfe et al. 1996). The mutated bacteria differentiated into bacteroids, which

were deviated in ultrastructure, prematurely degenerated, and did not fix nitrogen (Bialek et al. 1995). Some *Exo*[−] mutants of *R. leguminosarum* bv. *trifolii* induced noninfected nodules that contained neither infection threads nor bacteroids (Skorupska et al. 1995).

Whereas the genetic control of succinoglycan (EPSI) and galactoglucan (EPSII) in *Sinorhizobium meliloti* is well known (Becker et al. 1993a, 1993b, 1997; Buendia et al. 1991; Glucksmann et al. 1993a, 1993b; Müller et al. 1993; Reuber and Walker 1993), relatively little is known of the genetic control of synthesis and regulation of EPS in *R. leguminosarum* biovars. Until the present time, the *pssA* gene probably encoding the first glycosyl transferase in EPS synthesis was characterized in *R. leguminosarum* (Borthakur et al. 1986, 1988; Ivashina et al. 1994; van Workum et al. 1997). Three other genes, *pssCDE*, involved in EPS biosynthesis in *R. leguminosarum* bv. *trifolii* 5599 were identified (van Workum et al. 1997). Recently, the biochemical function of *pssDE* genes of *R. leguminosarum* was identified as glucuronosyl-(β 1→4)-glucosyl transferase (Pollock et al. 1998). The *pssC* gene was characterized as coding for a glucuronosyl-(β 1→4)-glucuronosyl transferase (Pollock et al. 1998). The *exoB* gene encoding the UDP-glucose 4-epimerase was identified in *R. leguminosarum* bv. *trifolii* (Sanchez-Andujar et al. 1997). Previously, the *psi* and *psr* genes, located on pSym plasmid of *R. leguminosarum* bv. *phaseoli*, which are engaged in the regulation of EPS biosynthesis, have been described (Borthakur et al. 1985; Borthakur and Johnston 1987).

In this paper, the sequence and genetic organization of the three closely linked genes *pssC*, *pssD*, and *pssE* of *R. leguminosarum* bv. *trifolii* TA1 is reported. In addition, in the work reported herein, the *exo133::Tn5* (*pssD133*) mutant of TA1, which does not produce detectable amounts of acidic EPS and induces the non-nitrogen-fixing nodules on clover, was microscopically characterized.

Previously, three genes, designated *prsD*, *prsE* (protein secretion), and *orf3*, were identified on the 5.26-kb *Pst*I-Bgl/II fragment adjacent to the *exo133* mutation in cosmid pARF136

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Nucleotide and/or amino acid sequence data are to be found at GenBank or EMBL as accession no. AF014054.

carrying genomic DNA of *R. leguminosarum* bv. *trifolii* TA1 (Król and Skorupska 1997). The DNA sequence of the neighboring 4.4-kb *Bgl*III-*Eco*RI fragment revealed three open reading frames (ORFs) that read toward the *Bgl*III site and were identified as *pssC*, *pssD*, and *pssE* (Fig. 1). The Tn5 insertion in the *exo133* mutant deficient in EPS synthesis was located in the middle of the *pssD* coding region (position 1777 to 1778 bp). Recently, nearly identical genes of the *R. leguminosarum* bv. *trifolii* strain 5599 (van Workum et al. 1997) and *R. leguminosarum* bv. *viciae* (J. A. Downie, Norwich; EMBL accession no. Y12758) were identified. Whereas the *pssCDE* genes of strain TA1 were highly homologous to *pssCDE* of other strains, they differed in gene lengths and gene overlaps (GenBank/EMBL accession no. AF014054). The overlapping genes indicate that expression of *pssC*, *pssD*, and *pssE* could be coupled. The proteins related to PssCDE, identified by a sequence homology search, showed the greatest similarity to prokaryotic glycosyl transferases (Table 1).

Mutant-Rt133-induced, non-nitrogen-fixing, white nodules on *Trifolium pratense* appeared 7 to 10 days later than nodules induced by the wild-type strain TA1. This was complemented by both EPS synthesis and nodulation deficiency by cosmid pARF136 and its subclones pARF1368 and pHJ. For the further mutational and transcriptional analysis of the *pssDE* genes, a promoter-less *lacZ*-Gm^r interposon (Becker et al. 1995) was cloned, in both orientations, into the single *Hind*III or *Bgl*III sites of plasmid pHJ (Fig. 1; Table 2). The *pssD*-*lacZ* and *pssE*-*lacZ* fusions, in both orientations, were introduced by marker exchanges into the wild-type strain TA1, resulting in the *R. leguminosarum* bv. *trifolii* strains TAH11A,B and TAB81A,B, respectively (Table 3). The TAH11A and TAH11B mutants with *lacZ*-Gm^r interposon located in the 5' end of *pssD*, independently of the interposon orientation, showed decrease in the nitrogenase activity measured by the reduction of acetylene (Hardy et al. 1982), compared with the wild-type activity (Table 4). The green mass of clover infected

Fig. 1. Genetic organization of *pss* genes involved in exopolysaccharide synthesis of *Rhizobium leguminosarum* bv. *trifolii* TA1. **A**, Restriction map of a 7.54-kb *Eco*RI fragment of pARF136 insert. Insertions of the *lacZ*-Gm^r interposon, shown as flags, indicate transcription direction of the promoterless *lacZ* gene. Names of *Rhizobium* mutants are inserted. **B**, The identified *prsDEorf3* and *pssCDE* genes. Bold arrows indicate orientation of transcription, rbs = putative ribosome binding sites. The head arrow indicates site of Tn5 insertion in Rt133 *exo* mutant. **C**, Several restriction fragments subcloned on the plasmids with names of the constructs.

Table 1. Homologies of the proteins predicted from open reading frames (ORFs) of the *pssCDE* genes of *Rhizobium leguminosarum* bv. *trifolii* TA1

Pss protein	Predicted protein (amino acids/kDa)	Proposed function of homologous protein (% identity)	Bacteria	Reference or EMBL accession no.
PssC	384/43.5	PssC glycosyl transferase (91%) PssC glycosyl transferase (91%) ExoM β -1,4 glycosyl transferase (27%)	<i>R. leguminosarum</i> bv. <i>trifolii</i> 5599 <i>R. leguminosarum</i> bv. <i>viciae</i> <i>R. meliloti</i>	van Workum et al. 1997 Y12758 Becker et al. 1993a
PssD	280/31.1	PssD glycosyl transferase (87%) PssD glycosyl transferase (86%) Cps14F enhancer glycosyl transferase activity (26%) SpsK glycosyl transferase (30%) EpsE glycosyl transferase (29%)	<i>R. leguminosarum</i> bv. <i>viciae</i> <i>R. leguminosarum</i> bv. <i>trifolii</i> 5599 <i>Streptococcus pneumoniae</i> <i>Sphingomonas</i> S88 <i>Lactococcus lactis</i>	Y12758 van Workum et al. 1997 Kolkman et al. 1997 Yamazaki et al. 1996 van Kranenburg et al. 1997
PssE	195/21.3	PssE glycosyl transferase (90%) PssE glycosyl transferase (90%) EpsF glycosyl transferase (36%) Cps14G β -1,4 galactosyl transferase (37%) SpsK glycosyl transferase (28%)	<i>R. leguminosarum</i> bv. <i>trifolii</i> 5599 <i>R. leguminosarum</i> bv. <i>viciae</i> <i>Lactococcus lactis</i> <i>Streptococcus pneumoniae</i> <i>Sphingomonas</i> S88	van Workum et al. 1997 Y12758 van Kranenburg et al. 1997 Kolkman et al. 1997 Yamazaki et al. 1996

with these mutants differed insignificantly from the clover inoculated with the wild-type TA1 strain. The EPS production was 25% higher than the wild-type level when the cassette was in the sense orientation and about 20% lower when interposon was in the antisense orientation (Table 4). The β -galactosidase activity in the mutant TAH11B in the sense orientation of *lacZ*-Gm^r was 4.8-fold higher than in the antisense orientation (167.2 and 34.8 Miller units, respectively), indicating the presence of the promoter upstream of *Hind*III site. The pJH1 plasmid, which is *Hind*III-*Pst*I subclone of pJH (Fig. 1) cloned in an opposite orientation to the *lacZ* promoter of the vector, did not complement the *exo133* mutation in *pssD*. On the other hand, pJS1 plasmid containing the *Sac*I fragment of pJH cloned in both orientations complemented this mutation. These data indicated that promoter of *pssD* is located between *Sac*I and *Hind*III sites, at the 5' end of the gene. The EPS production in the mutants with the interposon inserted in *Hind*III site (Table 4) indicated that the ORF of the *pssD* gene could start from the alternative ATG codon located downstream (59 bp) of the *Hind*III site. The AGGAGA ribosome binding site was identified 9 bp upstream of the second ATG start codon of *pssD* in accordance with data described by van Workum et al. (1997).

The *lacZ*-Gm^r insertion at *pssE* did not significantly affect the EPS synthesis and symbiotic phenotype of TAB81A and TAB81B mutants (Table 4). These results indicated that *PssE* may be not required for EPS production or that the mutation at

the 3' end of *pssE* did not affect the activity of this protein. The β -galactosidase activity of the *pssE-lacZ* fusion constructed in the direction of transcription was twofold higher than in the opposite direction (52.8 and 26.9 Miller units, respectively), indicating that the *pssDE* genes are under the same promoter.

To study in more detail the symbiotic defect revealed by *pssD133* mutation, the clover was inoculated with rhizobia, root nodules processed for light and electron microscopy as described earlier (Łotocka et al. 1997). In the nodules induced

Table 4. Exopolysaccharide (EPS) production and symbiotic properties of *Rhizobium leguminosarum* bv. *trifolii* derivatives^a

Strain	Mutation	Total EPS (μg/ml) ^b	Nodule/plant (no.)	Green wet mass/plant (mg)	Acetylene reduction (nM ethylene/h/plant)
TA1	Wild type	527 ± 86	12.3 ± 4.5	76.8 ± 9	74.7 ± 16
Rt133	<i>pssD</i> ::Tn5	0	4.6 ± 1	40.3 ± 5	0
TAH11A	<i>pssD-lacZ</i>	426 ± 27	16.5 ± 4.6	60.8 ± 7.7	34.0 ± 7
TAH11B	<i>pssD-lacZ</i>	657 ± 93	10.5 ± 2.5	66.4 ± 8	54.2 ± 10
TAB81A	<i>pssE-lacZ</i>	465 ± 43	11.9 ± 1.8	54.6 ± 6.5	61.8 ± 15
TAB81B	<i>pssE-lacZ</i>	586 ± 48	9.4 ± 0.5	60.0 ± 8.2	47.8 ± 11

^a Symbiotic properties of rhizobia were measured 28 days after inoculation. Values given are averages of at least four independent experiments with 20 plants for each treatment.

^b Amount of EPS is expressed as glucose equivalents (Loewus 1952).

Table 2. Plasmids and phages used

Plasmid/phage	Relevant characteristics	Reference
pRK7813	IncP, <i>mob</i> , Tra ⁻ cosmid vector, Tc ^r	Stanley et al. 1987
pH78	pRK7813 carrying MCS without <i>Hind</i> III site	This work
pSUP202	pBR325 derivative, <i>mob</i> , Cm ^r Tc ^r Ap ^r	Simon et al. 1983
pPH1JI	IncP, Tra ⁺ , Gm ^r Sp ^r	Beringer et al. 1978
pHI5	pPH1JI::Tn5, Tra ⁺ deleted derivative, Km ^r Gm ^r Sp ^r	This work
pBBR1MCS-3	<i>mob</i> , Tc ^r cloning vector	Kovach et al. 1995
pBBR1MCS-5	<i>mob</i> , Gm ^r cloning vector	Kovach et al. 1995
pARF136	pLAFR3 with 9-kb insert of TA1 complementing <i>exo133</i>	Skorupska et al. 1991
pARF1368	pRK7813 with 5.6-kb <i>Bam</i> HI fragment of pARF136 complementing <i>exo133</i>	Skorupska et al. 1991
pAB2001	Promoterless <i>lacZ</i> -Gm ^r interposon	Becker et al. 1995
pJH	pH78 with a 3,036-bp <i>Pst</i> I fragment of pARF1368	This work
pJH1	pBBR1MCS-5 with a 1,796-bp <i>Hind</i> III- <i>Pst</i> I fragment of pJH	This work
pJS1	pBBR1MCS-3 with a 1,477-bp <i>Sac</i> I fragment of pJH	This work
pHJH1	pJH derivative with <i>lacZ</i> -Gm ^r interposon in <i>Hind</i> III site	This work
pHJH2	pJH with <i>lacZ</i> -Gm ^r in opposite direction as in pHJH1	This work
pSu11	pSUP202 with insert from pHJH1 in <i>Pst</i> I site of vector	This work
pSu27	pSUP202 with insert from pHJH2 in <i>Pst</i> I site of vector	This work
pJB4	pJH derivative with <i>lacZ</i> -Gm ^r interposon in <i>Bgl</i> II site	This work
pJB8	pJH with <i>lacZ</i> -Gm ^r in opposite direction as in pJB4	This work
M13mp	Sequencing vector	Sambrook et al. 1989

Table 3. Bacterial strains used

Strain	Relevant characteristics	Reference
<i>Rhizobium leguminosarum</i> bv. <i>trifolii</i>		
TA1	Wild type, <i>str</i> rif	M. Djordjevic
Rt133	TA1, <i>pssD133</i> ::Tn5 (<i>exo133</i>)	Król and Skorupska 1997
TAH11A, B	TA1, <i>lacZ</i> -Gm ^r , cassette inserted in <i>Hind</i> III site of <i>pssD</i> in the opposite direction (A) and in the direction (B) of transcription	This work
TAB81A, B	TA1, <i>lacZ</i> -Gm ^r cassette inserted in the <i>Bgl</i> II site of <i>pssE</i> in the opposite direction (A) and in the direction (B) of transcription	This work
<i>Escherichia coli</i>		
JM101	<i>supE thi Δ(lac-proAB) F'</i> [<i>traD36 proAB⁺ lacI^q lacZΔM15</i>]	Sambrook et al. 1989
DH5α	<i>supE44 ΔlacU169 (φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Sambrook et al. 1989

by the Rt133 mutant, except for the meristematic zone, none of the developmental zone typical for cylindrical nodules was observed (Vasse et al. 1990) (Fig. 2A). Immediately below the meristem, a very narrow zone of cells, much larger than the meristematic ones, and rare, very large, infection threads were observed. Even though the enlarged threads have been found mainly at the intercellular spaces, they also penetrated the plant cells and then the major part of the plant cell was filled by the infection threads. The thread-containing cells were poorly vacuolated, without flattened and parietally located amyloplasts characteristic for the bacteroidal tissue. Close to the infection threads, large clusters of the bacteria were seen (Fig. 2B). The zone, which contained the infection threads, was always very narrow in the nodule induced by the Rt133

mutant. Basipetally to that zone, there was a large area of uniform parenchyma with vacuolated cells containing numerous, round amyloplasts with starch grains (Fig. 2A). The parenchyma adjoined directly to the nodule endodermis, and in the infection zone the endodermis was separated from the atypical bacteroidal cells with a thin layer of uninfected, small cells of inner cortex. The parenchyma zone has no counterpart in the wild-type nodule, in which that space was occupied by the bacteroidal tissue and the inner cortex.

The infection threads were tightly packed with bacteria and the thread matrix was compressed into the narrow film between bacteria (Fig. 3A–C). The thread wall was not uniformly fine-fibrillar, as in the wild-type nodules, and clear, osmophilic, discontinuous layers were observed in the thread

Fig. 2. Semithin section of 4-week-old clover root nodule induced by the *Rhizobium leguminosarum* bv. *trifolii* Rt133 Exo⁻ mutant. **A** and **B**, Nodule anatomy as seen on longitudinal section: M = meristematic zone; VB = vascular bundle; P = parenchyma; thick arrow = infection thread in enlarged intercellular space; arrowheads = cells penetrated with infection threads; white asterisk = clusters of bacteria after releasing from the infection threads. Bar = (**A**) 100 and (**B**) 32.60 μ m.

Fig. 3. Ultrastructure of infection threads in 4-week-old clover nodule induced by *Rhizobium leguminosarum* bv. *trifolii* Rt133. **A**, Large infection thread inside a host cell. Location of cells shown here was submeristematic. **B**, An area suggesting breakage of infection thread wall integrity and releasing of bacteria from infection thread into the cytoplasm: IT = infection thread; thick arrows = thread wall; arrows = thread matrix; arrowhead = vesicle with osmiophilic content; white rosettes = bacteria. **C** and **D**, Ultrastructure of infection thread wall: B = bacteria; thick arrows = thread wall; white arrows = homogenous inserts between osmiophilic layers of thread wall material. **E**, Symbiosomes in host cell: CW = cell wall; N = cell nucleus; asterisks = bacteroids. Bars = (A) 3.15, (B) 1.25, (C) 1.65, (D) 0.39, and (E) 1.24 μ m.

walls of Rt133 nodules (Fig. 3C–D). Between the layers, homogenous and less osmiophilic material was inserted. In the cytoplasm surrounding the infection threads, large vesicles with osmiophilic content were always seen (Fig. 3B). The infection thread walls with abnormal morphology and the vesicles containing the deposited material can presumably be the result of an induced plant defense response elicited by defective nodulation of the Rt133 mutant. The typical bacteria release from unwallied infection threads was not observed. Instead, it was often observed that the bacteria got out of the infection threads through the thread-wall discontinuity (mechanical breach or enzymatic digestion?) (Fig. 3B). After releasing, the bacteria were surrounded by thread matrix, but symbiosomes were observed very rarely, assuming a defect in the endocytosis process (Fig. 3E). At the ultrastructural level, the nodules induced by the Rt133 mutant that contained the enlarged infection threads with abnormal walls and densely packed bacteria resembled the nodules induced after long delay by *R. meliloti* 0540 carrying the mutation in the *exoY* gene (Niehaus et al. 1993). The difference between them was the way the bacteria released from the infection thread and the absence of the numerous symbiosomes into the host cytoplasm in the nodules induced by Rt133.

The conclusion that the *pssCDE* genes of *R. leguminosarum* bv. *trifolii* strain TA1, otherwise the same genes of strain 5599 described by van Workum et al. (1997), are significantly overlapping would suggest the coupled translation. This difference could be explained by the different geographical origin of both strains. The TA1 strain is an Australian strain described as defective in nodulation of *Trifolium subterraneum* cv. Woogenellup in unfavorable environmental conditions (Lewis-Henderson and Djordjevic 1991). The RBL5599 strain is a European strain with marked pSym5 introduced into the cured pSym *R. leguminosarum* bv. *trifolii* LPR5 strain (van Workum et al. 1997).

The *pssD* gene, in the longest possible ORF, overlaps *pssC* and *pssE* genes, but the genetic complementation of the *exo133* mutation and production of EPS in *R. leguminosarum* bv. *trifolii* mutated in the N-terminal part of the PssD protein indicates the possibility that PssD starts from the alternative ATG codon. The Tn5 insertion in the central region of *pssD* totally abolished the EPS production and the *R. leguminosarum* bv. *trifolii* Rt133 mutant induced non-nitrogen-fixing nodules on clover. From these results, one could conclude that *pssD* gene encoding for glycosyl transferase is required for biosynthesis of EPS. The *pssD::Tn5* (*exoIII*) mutant described by van Workum et al. (1997) is located 43 bp upstream of *pssD133* and displayed similar phenotype in respect of EPS production and symbiosis with clover. Transcriptional fusion *pssD-lacZ* and the complementation data with the restriction fragments containing the deleted 5' end of *pssD* indicated the presence of the promoter for *pssDE* genes upstream of the *HindIII* site. The *pssD* gene of TA1 in the shorter form is 162 bp longer at the 3' end than the *pssD* of strain 5599 and significantly overlaps the *pssE*. The level of β -galactosidase activity in the transcriptional fusions of *pssE-lacZ* indicated the presence of the common promoter for *pssDE* genes. The respective homogenotes of *R. leguminosarum* bv. *trifolii* did not reveal symbiotic defects, and EPS production is comparable to the wild-type level. Therefore, the putative function of PssE as glycosyl transferase in EPS biosynthesis can be pro-

posed only based on the sequence homology to known proteins. The latest experimental data showed the common action of PssDE as glucuronosyl transferase in acidic EPS biosynthesis of *R. leguminosarum* bv. *trifolii* 5599 (Pollock et al. 1998).

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