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Molecular characterization and symbiotic importance of *prsD* gene of *Rhizobium leguminosarum* bv. *trifolii* TA1[o]

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The *prsD, prsE* and *orf3* genes of *Rhizobium leguminosarum* bv. *trifolii* strain TA1 encode the proteins which are significantly related to the family of bacterial ABC transporters type I secretion systems. The *prsD*:Km^r mutant of strain TA1 induced non-nitrogen-fixing nodules on *Trifolium pratense*. Microscopic analysis of the nodules induced by *prsD* mutant did not reveal major abberations in the bacteroid appearance. The exopolysaccharide of *prsD* mutant was produced in increased amount and its level of polymerization was changed. SDS/PAGE of the proteins from the culture supernatants showed a lack of the 47-kDa protein in the culture of *prsD* mutant. Thus, PrsD may play a role in the export of this protein.

Rhizobial exopolysaccharides (EPS) play a significant role in establishment of effective symbioses between rhizobia and legumes which form indeterminate nodules (Leigh & Walker, 1994). The bacterial mutants which are defective in EPS production elicit non-nitrogen fixing nodules that are not invaded by bacteria as are nodules induced by *R. meliloti* mutants (Finan *et al.*, 1985), or only partially infected as are nodules induced by *R. leguminosarum* bv. *trifolii* mutants (Chen *et al.*, 1985; Deryło *et al.*, 1986; Białek *et al.*, 1995). A number of roles have been suggested for acidic EPS in symbioses, especially in mediating the invasion of root nodules (Leigh & Walker, 1994; Skorupska *et al.*, 1995) or in

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Abbreviation: EPS, exopolysaccharides.

avoiding recognition responses of the plant defence system by masking the potential elicitors on bacterial surface (Niehaus *et al.*, 1993).

In our earlier studies, we described three genes of *R. leguminosarum* bv. *trifolii* TA1 designated *prsD, prsE* (protein secretion) and *orf3* (Król & Skorupska, 1997). The *prsDE* genes shared significant homology with the genes encoding ABC transporter proteins PrtDE from *Erwinia chrysanthemi* (Lettofé *et al.*, 1990) and AprDE from *Pseudomonas aeruginosa* (Duong *et al.*, 1992), but the detailed function of *prs* genes was not established. Simultaneously, the homologous genes encoding the protein export system in *R. leguminosarum* bv. *viciae* (Finnie *et al.*, 1997) and *R. meliloti* (York & Walker, 1997) were described. In *R. leguminosarum* bv. *viciae,* the *prsDE* genes were required for the secretion of the nodulation protein (NodO), extracellular endoglycanases PlyA, PlyB and other proteins which are necessary for symbiotic nitrogen fixation (Finnie *et al.*, 1997; 1998). Additionally, the *exsH* encoding a homologue of endo-1,3-1,4-β-glycanases with glycine-rich nonameric repeats, secreted by a type I secretion system composed of PrsD and PrsE, was identified in *R. meliloti* (York & Walker, 1997).

In this paper, we characterized the *prsD* mutant, disrupted in the middle of ORF by insertion of the Km^r cassette, in respect of symbiotic properties and EPS production. We also cloned the region preceding the *prsDE* and *orf3* genes, to study the transcriptional activity of these genes, by fusion of the *prsD* and orf3 promoters with promoterless *lacZ* gene. The putative function of PrsD in *R. leguminosarum* bv. *trifolii* is discussed.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* DH5α and derivative strains were grown in LB medium at 37°C (Sambrook *et al.*, 1989). *R. leguminosarum* strains were grown on 79CA medium at 28°C (Vincent, 1970) or Brown and Dilworth minimal medium (1975). Antibiotics were supplemented as required at the following concentrations (μg/ml) for *E. coli* and *R. leguminosarum*: rifampicin, 40; tetracycline, 10; kanamycin, 40; gentamicin, 5, and ampicilin, 100.

Plant nodulation tests, light and electron microscopy. *R. leguminosarum* bv. *trifolii* were assayed for their symbiotic properties on *Trifolium pratense* cv. Ulka as described earlier (Białek *et al.*, 1995). Four week-old nodules were prepared for light and electron microscopy as described by Łotocka *et al.* (1997).

Analysis of extracellular proteins in the culture supernatants. Rhizobia were grown for 48 h at 28°C in 100 ml of Brown and Dilworth minimal medium with glycerol as a carbon source. Proteins were isolated from supernatants by precipitation in the presence of ammonium sulphate (80%) and separated by SDS/PAGE (Sambrook *et al.*, 1989).

Exopolysaccharide analysis. EPS was precipitated from 1 ml of the supernatants of 5 day-old cultures growing on 79CA medium containing glycerol, by adding 3 vol. of ethanol. For quantitative analysis of the total sugars concentration in EPS the anthrone method was used (Loewus, 1952). Concentration of reducing sugars was measured by the method of Lever (1972). The amounts of sugars were given as equivalents of glucose. Viscosity of the culture was measured as described by Finnie *et al.* (1997).

For the detection of endoglycanase activity in TA1 and TA1.34, to the Brown and Dilworth minimal agar medium precipitated EPS or carboxymethyl cellulose (CM) were added as described by Finnie *et al.* (1997). After 3 days colonies were washed off and the plates were stained with 0.1% Congo Red (Mateos *et al.*, 1992).

DNA manipulation and bacterial matings. Recombinant DNA techniques such as restriction analysis, cloning procedures and

transformation were carried out according to Sambrook *et al.* (1989). Plasmids were transferred from *E. coli* to *R. leguminosarum* bv. *trifolii* TA1 using triparental matings (Ditta *et al.*, 1980). The double mutant Rt56SupEPI was constructed as follows: the fragment containing Gm^r cassette from pMS272 (Becker *et al.*, 1995) was cloned into single *Bam*HI site of pAM5 plasmid (Fig. 1). Then *Eco*RI-*Pst*I fragment (3.2 kb) of the pAM5 derivative was recloned on the suicide plasmid pSUP202 (Simon *et al.*, 1983), and introduced by triparental mating into Rt56 (Skorupska *et al.*, 1995). Transconjugants after the marker exchange were selected on Tc^s Gm^r. The homologous recombination was verified by Southern hybridization.

Assays for β-galactosidase activity were performed according to Miller (1972).

The accession numbers for the sequence described in this paper are U44387, X98117 and AF014054.

RESULTS AND DISCUSSION

Symbiotic phenotype of *prsD* mutant of *R. leguminosarum* bv. *trifolii* TA1

Previously, we constructed a *prsD* mutant by the insertion of kanamycin resistance cassette in *Bam*HI site of the ORF and the mutated insert was introduced into the genome of wild-type strain TA1 by marker exchange, resulting in the mutant designated TA1.34 (Fig. 1) (Król & Skorupska, 1997). The strain TA1.34 elicited nodules on clover (*T. pratense*) without delay, but the number of nodules was higher (16.7/plant) than in the parental strain (12.7/plant). The nodules were white and measurement of the acetylene reduction indicated that the nodules did not fix nitrogen. The symbiotic phenotype of TA1.34 was essentially the same as described for a *prsD*::Tn5 mutant of *R. leguminosarum* bv. *viciae* (Finnie *et al.*, 1997).

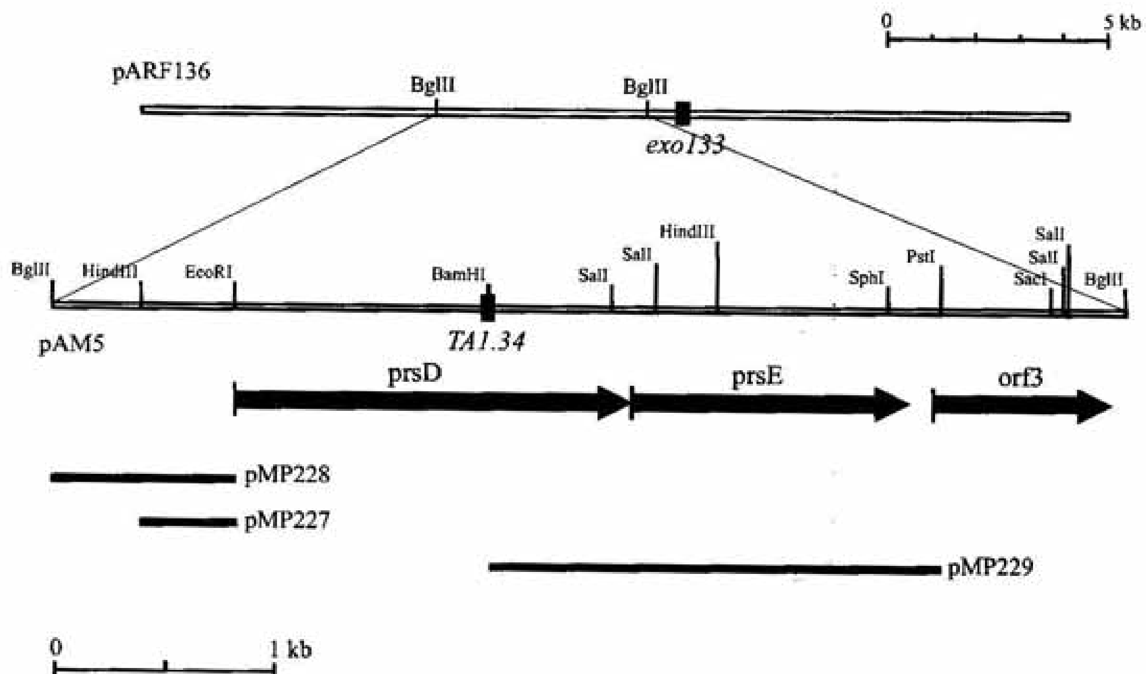

Figure 1. Map of the 4.79 kb *Bgl*II fragment carring the *prsDEorf3* genes of *R. leguminosarum* bv. *trifolii* TA1.

The insertions of Tn5 transposon in cosmid pARF136, giving *exo133* and Km^r cassette in *prsD* gene of pAM5, giving TA1.34 mutation (Król & Skorupska, 1997) are shown as black rectangles. The bold arrows indicate the direction of transcription, rbs – putative ribosome binding sites. At the bottom, the subclones of pAM5 in pMP220 vector are shown.

The root nodules induced by both wild-type, as well as TA1.34 mutant strains were cylindrical and identical in their anatomical structure. Both contained the apical meristem with dividing cells, infection zone, interzone II/III and the fixation zone, as described by Vasse *et al.* (1990) for indeterminate nodules. The nodules induced by strain TA1.34 examined under electron microscope, revealed small ultrastructural differences in comparison to those induced by the wild-type. In mutant-induced nodules, the amyloplasts present in the uninfected cells of bacteroidal tissue contained more of starch grains (not shown) than in TA1 induced nodules. The symbiosomes, in the fixation zone of mutant nodules, had much narrowed peribacteroidal spaces (Fig. 2 b). The vesicles present in the cytoplasm of differentiated mutant bacteroids were filled with fine-fibrillar, osmiophilic substance (Fig. 2 b), while the vesicles in wild-type bacteroids were electron-transparent with only minute content (Fig. 2 a). Similar vesicles in the bacteroids of subterranean clover nodules were earlier described (Dart & Mercer, 1963).

Characterization of *prsD* mutant

The strain TA1.34 was growing on mannitol-yeast-extract medium slower than the wild-type strain, what was clearly seen after 24 h when the absorbance of TA1.34 was about

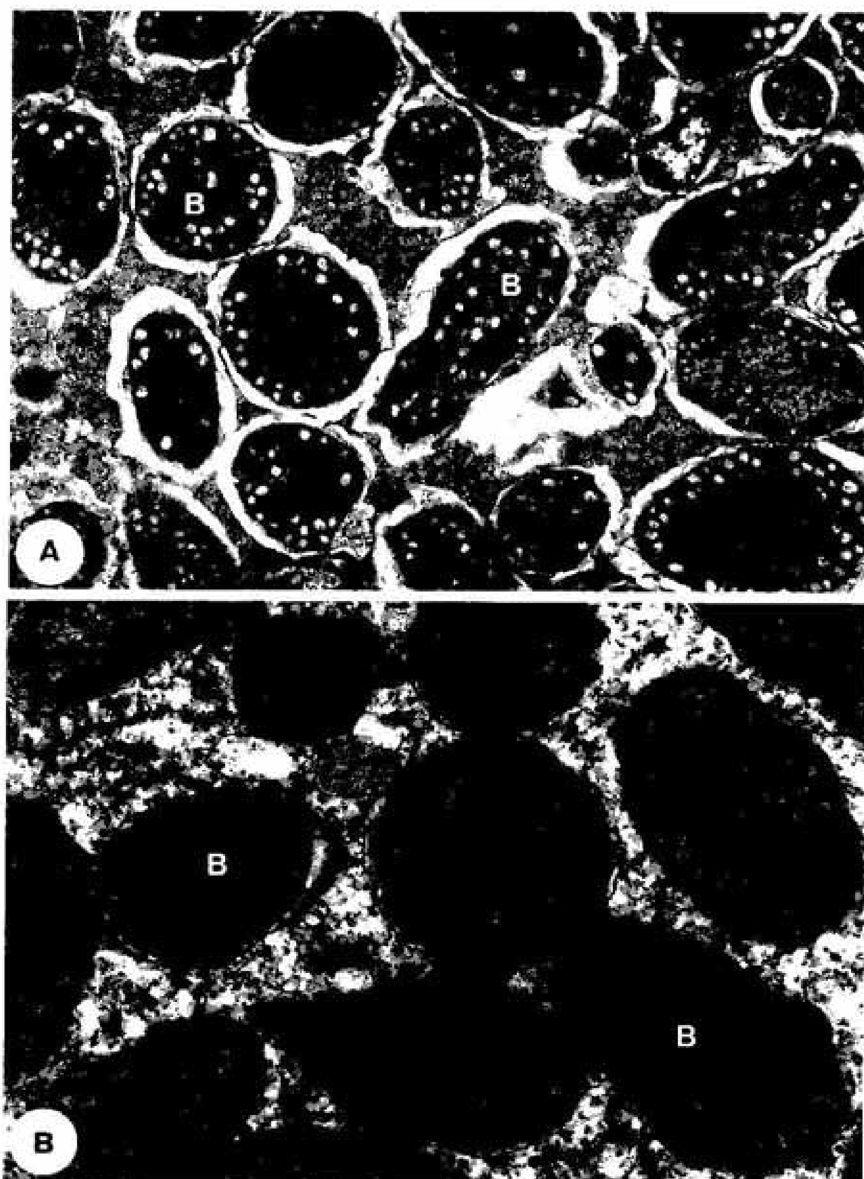

Figure 2. Ultrastructure of the symbiosomes in the fixation zone of 4 week-old clover nodule induced by (A) *R. leguminosarum* bv. *trifolii* TA1 and (B) TA1.34 mutant strain.

B, bacteroid; arrow, vesicles in the bacteroid cytoplasm; arrowhead, peribacteroidal space; double arrowhead, peribacteroidal membrane; asterisk, host cytoplasm. Magnification: (A) 14000×, (B) 25000×.

50% in comparison with the isogenic control strain. This mutant formed mucoid colonies on the 79CA agar medium and the amount of acidic EPS in the culture supernatants was much higher than in the case of control strain (995 μg/ml and 646 μg/ml, respectively). The lower concentration of reducing sugars (102 μg/ml in comparison to 192 μg/ml in TA1 strain) and 2.6 times higher viscosity of the culture supernatants of the strain TA1.34 pointed to changes in EPS polymerization in the mutant strain. We concluded from these experiments that the putative endoglycanase, which normally cleaved the EPS, was absent from the TA1.34 culture supernatant. Then, we examined the endoglycanase activity in wild-type and *prsD*:Km^r strains. In several plate tests (Mateos *et al.*, 1992; Finnie *et al.*, 1997; York & Walker, 1997) we could not demonstrate any endoglycanase activity in wild-type strain TA1 and the mutant TA1.34. It is possible that the activity of the putative glycanase which is exported by PrsDE transport system of *R. leguminosarum* bv. *trifolii* TA1 is predominantly cell associated and it could not be detected by the plate tests.

In the 4.0 kb sequence preceding the *prsD* gene we have not found the ORF for endoglycanase described as PlyA in *R. leguminosarum* bv. *viciae* (Finnie *et al.*, 1998). Instead, we found only residual sequences which were homologous to *plyA*. We concluded, that in strain TA1, the putative endoglycanase is not linked to *prsDE* genes. To check whether PrsD may mediate secretion of any protein, we compared the SDS/PAGE profiles of proteins prepared from supernatants of *exo56* and *exo56prsD*:Gm^r strains (Fig. 3). We used *exo56* strain, which fails to produce EPS, to improve the efficiency of removal of the cells from supernatants. One protein (47 kDa) which is clearly present in *exo56* supernatant, is absent from in *exo56prsD*:Gm^r mutant. We consider it possible that this 47-kDa protein lacking in the supernatant of *prsD* mutant, is exported by *prsDEorf3* type I system. The 47-kDa protein could be the protein which is important for the symbiotic nitrogen fixation, because the TA1.34 is a non-fixing mutant. To elucidate the function of this protein in *R. leguminosarum* bv. *trifolii* TA1 further experiments are needed.

Transcription analysis of *prsDEorf3* genes

The 4.8 kb *Bgl*II fragment of pARF136, containing the *prsDprsEorf3* genes, was cloned into pUC19 vector, giving the pAM5 plasmid. The promoter activity in the pAM5 insert was tested by subcloning *Bgl*II/*Eco*RI and *Hin*dIII/*Eco*RI fragments preceding the *prsD* gene, in front of the promoterless *lac*Z gene in pMP220 (Spaink *et al.*, 1987), giving pMP228 and pMP227, respectively (Fig. 1). These plasmids were mobilized into *E. coli* DH5α, *R. leguminosarum* bv. *trifolii* TA1 and Exo$^-$ mutant Rt133 (Król & Skorupska, 1997) and the level of *lacZ* expression was measured. Both cloned fragments, 0.8 kb and 0.4 kb, tested for pro-

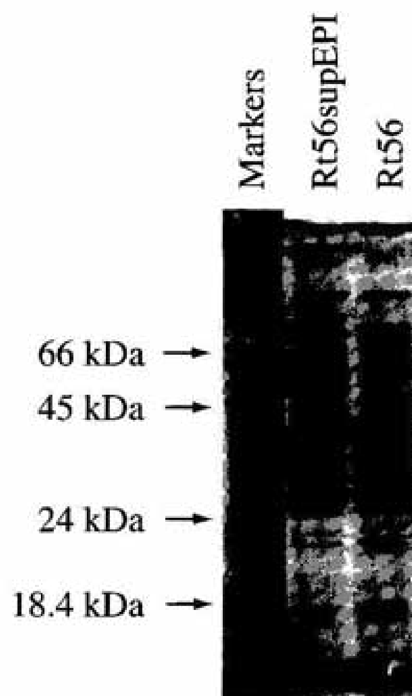

Figure 3. SDS/PAGE separation and Coomassie Blue staining of extracellular proteins prepared from *R. leguminosarum* bv. *trifolii* culture supernatants.

The lines and numbers indicate positions of molecular mass markers.

Table 1. ***β*-Galactosidase activities expressed by pMP220 with promoters of *prsD* or *orf3* cloned in front of the promoterless *lacZ* gene**

Plasmid	*E. coli* DH5*α*	RtTA1 (wild-type)	Rt133 (*pssD133*)
pMP220	0	82.5	83.4
pMP224	72.24	974.7	818.5
pMP228	59.57	678.0	719.1
pMP229	5.23	383.18	n.t.

β-Galactosidase activities are given in Miller units as averages of three or four independent assays. The background of *β*-galactosidase of *E. coli* strain was 0.4 ± 0.1, of RtTA1 20.2 ± 5 and of Rt133 15.2 ± 3 Miller units; n.t., not tested

moter activity showed increased *β*-galactosidase activity on *E. coli* DH5*α*, *R. leguminosarum* bv. *trifolii* TA1 and Rt133 backgrounds, when either was cloned in the direction towards the *prsD* reading frame (Fig. 1, Table 1). The level of transcription was not essentially changed in Exo$^-$ mutant. The high expression of *lacZ* gene in pMP227 and pMP228 in *E. coli* and lack of the effect of flavone (rutin), on the expression of *lacZ* in rhizobia (not shown) indicate the presence of a non-symbiotic promoter in front of *prsD* gene, transcribed from left to right.

The *prsE* and *orf3* genes are separated by the 150 bp intergenic region (Król & Skorupska, 1997) and the presence of an additional promoter in this region was tested. The *Bam*HI-*Pst*I fragment containing the 3′ end of *prsD, prsE* and 5′ end of *orf3* genes was subcloned into pMP220 in front of the promoterless *lacZ,* giving the pMP229. The *β*-galactosidase activity was measured in *E. coli* DH5*α* and *R. leguminosarum* bv. *trifolii* TA1, harbouring the pMP229 (Table 1). Generally, the *lacZ* expression in pMP229 was lower than in pMP227 and pMP228 constructs, pointing to weak, but clearly measured promoter activity. In summary, the transcriptional analysis of *prsDEorf3* genes showed the presence of a strong promoter in front of *prsD* and a second, weak promoter preceding the *orf3*. It is possible that the *orf3* gene can form a separate transcription unit.

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