Identification of genes in *Rhizobium leguminosarum* bv. *trifolii* whose products are homologues to a family of ATP-binding proteins

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The specific interaction between rhizobia and their hosts requires many genes that influence both early and late steps in symbiosis. Three new genes, designated *prsD*, *prsE* (protein secretion) and *orf3*, were identified adjacent to the *exo133* mutation in a cosmid carrying the genomic DNA of *Rhizobium leguminosarum* bv. *trifolii* TA1. The *prsDE* genes share significant homology to the genes encoding ABC transporter proteins PrtDE from *Erwinia chrysanthemi* and AprDE from *Pseudomonas aeruginosa* which export the proteases in these bacteria. PrsD shows at least five potential transmembrane hydrophobic regions and a large hydrophilic domain containing an ATP/GTP binding cassette. PrsE has only one potential transmembrane hydrophobic domain in the N-terminal part and is proposed to function as an accessory factor in the transport system. ORF3, like PrtF and AprF, has a typical N-terminal signal sequence but has no homology to these proteins. The insertion of a kanamycin resistance cassette into the *prsD* gene of the *R. leguminosarum* bv. *trifolii* TA1 wild-type strain created a mutant which produced a normal amount of exopolysaccharide but was not effective in the nodulation of clover plants.

Keywords: *Rhizobium leguminosarum* bv. *trifolii*, transport system genes, ATP-binding proteins, *exo* region

INTRODUCTION

Rhizobium, *Bradyrhizobium* and *Azorhizobium* are bacterial genera that form nitrogen-fixing nodules on legume roots (Schulze *et al.*, 1994). *Rhizobium leguminosarum* bv. *trifolii* induces nitrogen-fixing nodules on the roots of its host plant, clover (*Trifolium* spp.). Regulatory (*nodD*), common (*nodABCII*) and host-specific (*nodFERL*, *nodMN*) nodulation (*nod*) genes are located on symbiotic plasmid pSym (Djordjevic *et al.*, 1985; Schofield *et al.*, 1986). Besides the nodulation and nitrogen-fixation genes, genes encoding extracellular polysaccharides (*exo*) and lipopolysaccharides (*lps*) are needed for successful nodulation (Leigh & Walker, 1994). Mutants of *R. l. trifolii* deficient in exopolysaccharide synthesis form nodules which do not fix nitrogen (Chen *et al.*, 1985; Deryło *et al.*, 1986; Białek *et al.*, 1995; Skorupska *et al.*, 1995).

In view of the close interactions that occur between *Rhizobium* and its host legumes it seems likely that products of several genes may be exported proteins. Transport systems are known which translocate substances across the two membranes of the Gram-negative bacterial cell envelope in a single step (Higgins, 1992; Fath & Kolter, 1993). Among the best characterized of these transporters are ABC-type (ATP-binding cassette) systems which export proteins such as haemolysin of *Escherichia coli*, proteases of *Erwinia chrysanthemi* and *Pseudomonas aeruginosa*, peptides such as colicin V and non-protein substrates such as *Klebsiella pneumoniae* capsular polysaccharide and *Agrobacterium tumefaciens* and *Rhizobium meliloti* β -1,2-glucans (for reviews see Fath & Kolter, 1993; Higgins, 1992). The secretion apparatus consists of a transmembrane protein with an ATP-binding domain and additional proteins located in the periplasm and in the outer membrane which function to facilitate the export of products through both membranes (Fath & Kolter, 1993).

Here we present the nucleotide sequence of three genes

The accession numbers for the sequence reported in this paper are U44387 (GenBank) and X98117 (EMBL).

from *R. l. trifolii* TA1: *prsD*, *prsE* (protein secretion) and *orf3*. PrsD is significantly related to the bacterial ABC exporter of proteases PrtD from *Erw. chrysanthemi* (Letoffé *et al.*, 1990) and AprD from *P. aeruginosa* (Duong *et al.*, 1992). The *prsE* structural gene was identified as the next gene linked to *prsD*, with homology to *prtE* and *aprE* genes encoding accessory factors in related export systems. The third gene, *orf3*, located immediately downstream of *prsE* is not homologous to PrtF or AprF outer-membrane factors.

METHODS

Bacterial strains, plasmids and growth conditions. The strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown in LB medium at 37 °C (Sambrook *et al.*, 1989). *R. l. trifolii* was grown in 79CA medium at 28 °C (Skorupska *et al.*, 1991). Antibiotics were added at the following concentrations ($\mu\text{g ml}^{-1}$) for *E. coli* and *R. l. trifolii*: tetracycline, 10; rifampicin, 40; kanamycin, 40. Exopolysaccharide production was detected as described by Becker *et al.* (1995). To assay protease activity, 79CA agar plates containing skimmed milk (1.5%) were used (Guzzo *et al.*, 1991). For testing cellulase activity, 79CA plates with carboxymethylcellulose (2%) were used and stained with Congo red (0.1%). To measure polygalacturonate lyase activity, 79CA plates containing polygalacturonic acid (0.25%) were used and stained with cetyltrimethylammonium bromide (1%) (Mateos *et al.*, 1992).

DNA manipulations and bacterial matings. Recombinant DNA techniques such as restriction analysis, cloning procedures, transformation, labelling of DNA and hybridization were carried out according to Sambrook *et al.* (1989). Plasmids

Fig. 1. Restriction map of the *prs* region from *R. l. trifolii* TA1. The genes *prsD*, *prsE* and *orf3* and the direction of their transcription are shown. Plasmid or phage subclones of pARF136 are shown at the bottom. The insertions of the transposon Tn5 *exo133* mutation into cosmid pARF136 and the *Km^r* cassette TA1.34 into pJ6 are marked.

were transferred from *E. coli* to *R. l. trifolii* TA1 using triparental mating (Skorupska *et al.*, 1991). Homologous recombination in *R. l. trifolii* TA1 was carried out with plasmid pPH1JI which is incompatible with the cosmid vector (Ruvkun & Ausubel, 1981). Homogenization was verified by Southern hybridization with pARF136 as a probe.

DNA sequencing. DNA sequencing reactions were performed by the dideoxy-termination method using M13mp18/mp19 vectors with [α - ^{32}P]dATP (Amersham) as the radioactive label. Sequenase version 2.0 (Amersham) was used according to the manufacturer's (USB) guidelines. Both strands of DNA were

Table 1. Strains, plasmids and phages used in this study

Strain, plasmid or phage	Relevant characteristics*	Reference or source
<i>R. l. trifolii</i>		
TA1	Wild-type bv. <i>trifolii</i>	M. Djordjevic
24.1	Wild-type bv. <i>trifolii</i> , <i>Sm^r</i>	Skorupska <i>et al.</i> (1991)
<i>E. coli</i>		
JM101	$\Delta(\text{lac-proAB}) \text{ supE thi (F' traD36 proAB lacI}^q\text{Z}\Delta\text{M1)}$	Sambrook <i>et al.</i> (1989)
DH5 α	<i>supE44</i> $\Delta\text{lacU169 (}\phi 80 \text{ lacZ}\Delta\text{M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1}$	Sambrook <i>et al.</i> (1989)
HB101	<i>F⁻ supE44 thi recA13 hsdS20 lacY1 rpsL20 (Sm^r)</i>	Sambrook <i>et al.</i> (1989)
Plasmids		
pRK7813	IncP, <i>mob</i> Tra ⁻ cosmid vector, Tc ^r	Stanley <i>et al.</i> (1987)
pRK2013	Helper plasmid, <i>Km^r</i>	Ditta <i>et al.</i> (1980)
pARF136	pLAFR3 clone with 19 kb insert of TA1	Skorupska <i>et al.</i> (1991)
pARF1368	5.6 kb <i>Bam</i> HI fragment of pARF136 cloned into pRK7813	Skorupska <i>et al.</i> (1991)
pJ6	2.19 kb <i>Eco</i> RI– <i>Hind</i> III fragment of pARF136 cloned into pRK7813	This study
Phages		
M13mp18, mp19	Sequencing vectors	Sambrook <i>et al.</i> (1989)
pJE1	4.0 kb <i>Bgl</i> II– <i>Eco</i> RI fragment of pARF136 cloned into M13	This study
pJ61	1.15 kb <i>Eco</i> RI– <i>Bam</i> HI fragment of pARF136 cloned into M13mp18	This study
p216	1.04 kb <i>Bam</i> HI– <i>Hind</i> III fragment of pARF1368 cloned into M13	This study
pM1	1.02 kb <i>Hind</i> III– <i>Pst</i> I fragment of pARF1368 cloned into M13	This study

* *Km*, kanamycin; *Se*, streptomycin; *Tc* tetracycline.

Fig. 2. Sequence alignments of (a) PrsD (*R. l. trifolii*), PrtD (*Erw. chrysanthemi*) and AprD (*P. aeruginosa*) and (b) PrsE (*R. l. trifolii*), PrtE (*Erw. chrysanthemi*) and AprE (*P. aeruginosa*). Conservative or identical amino acids in all three proteins are indicated by a black box. Amino acid residues are numbered on

sequenced. Computer analysis was performed by the Oxford Molecular Group, Oxford, UK, using DNASIS (Hitachi Software Engineering) and PC/GENE (IntelliGenetics). Gene sequences and corresponding amino acid sequences were used in homology searches of both DNA (GenBank and EMBL) and protein (SWISS-PROT) databases using the FASTA program (Pearson & Lipman, 1988). Multiple alignment was done with the MACAW program (Schuler *et al.*, 1991).

Plant tests. *R. l. 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). After 5 weeks the plants were checked for symbiotic parameters. Nitrogen fixation activity was determined by the acetylene reduction assay (Hardy *et al.*, 1982).

RESULTS

Subcloning of *prsD*, *prsE* and *orf3*

The recombinant cosmid clone pARF136 was identified by complementation of the *Exo*⁻ mutation (*exo93*) in *R. l. trifolii* TA1 (Skorupska *et al.*, 1991). By random mutagenesis of pARF136 with Tn5, we identified a Tn5 insertion, designated pKm133, which mapped in a 5.6 kb *Bam*HI fragment of pARF136 (Fig. 1). Recombination of this mutation into *R. l. trifolii* TA1 resulted in an *Exo*⁻ mutant phenotype. Clover infected with *R. l. trifolii* TA1 *exo133* formed non-nitrogen-fixing nodules.

DNA sequence analysis

The nucleotide sequence analysis of the 5.263 kb *Pst*II-*Bgl*II fragment adjacent to the *exo133* mutation (Fig. 1) revealed at least three ORFs; one, designated *prsD*, extends from an ATG initiation codon at nt 1256 to a TAG termination codon at nt 2968 and encodes a polypeptide of 570 aa with a deduced *M_r* of 60887. The sequence GAGG, which was found 2 bp further upstream, resembles a typical ribosome binding site. No obvious promoter sequence upstream of *prsD* was found.

A second ORF, *prsE*, 15 nt downstream of *prsD* starts from a GTG initiation codon at nt 2984 and encodes a polypeptide of 433 aa with a deduced *M_r* of 47146. No N-terminal signal sequence was identified in this protein. A potential ribosome binding sequence (AGAAGGA-AG) 2 bp upstream of GTG codon was identified.

A third ORF, *orf3*, 150 nt downstream of *prsE* initiates probably at the ATG codon at nt 4433 and encodes a polypeptide of 149 aa with a predicted *M_r* of 16502. A putative ribosome binding site was identified (AGGGT) 6 bp upstream of the initiation codon.

The right. The conserved Walker motifs are double underlined. The extents of putative transmembrane domains in PrsD and PrsE are overlined; the membrane-associated domain in PrsE is indicated by a dotted line.

Comparison of PrsD, PrsE and ORF3 with other bacterial secretion systems

A comparison of the deduced amino acid sequence of PrsD to proteins in the database revealed significant homologies to prokaryotic cytoplasmic membrane transporter proteins. Common structural features of these transport systems include an integral membrane domain and a highly conserved hydrophilic, ATP-binding domain with ATPase activity.

The hydropathy profile (Eisenberg *et al.*, 1984) of the PrsD protein revealed at least five hydrophobic regions, probably corresponding to transmembrane or membrane-associated regions (Fig. 2a). A putative ATP-binding site was found in the central region of the hydrophilic domain. The latter domain contains two Walker motifs required for the binding of ATP (Fig. 2a) (Walker *et al.*, 1982).

The most significant homology was to the *P. aeruginosa* AprD protein involved in the secretion of alkaline protease (Duong *et al.*, 1992) and to the *Erw. chrysanthemi* PrtD protein required for the export of protease (Letoffé *et al.*, 1990). The alignments of these proteins indicated that PrsD is 44.4% identical and 82.4% similar to AprD and 40.6% identical and 78.6% similar to PrtD (Fig. 2a).

The hydropathy profile of PrsE shows one N-terminal hydrophobic, transmembrane domain, a C-terminal membrane-associated globular domain and the lack of a signal peptide. This protein is homologous to the second component of this type of secretion system in *Erw. chrysanthemi* (PrtE, 27.8% identity, 71.4% similarity; Letoffé *et al.*, 1990) and in *P. aeruginosa* (AprE, 26.7% identity, 72.1% similarity; Duong *et al.*, 1992) (Fig. 2b). These proteins, referred to as accessory factors (AF; Fath & Kolter, 1993), are anchored in the inner membrane and span the periplasm. They probably act to connect the inner and outer membranes and facilitate the export of substrates through both membranes. PrtE is localized to both the inner and outer membrane (Delepelaire & Wandersman, 1991).

The predicted ORF3 product showed no sequence similarity to the outer-membrane proteins PrtF of *Erw. chrysanthemi* and AprF of *P. aeruginosa*, nor to other prokaryotic proteins. However, analysis of the deduced amino acid sequence of ORF3 revealed the presence at its N terminus of a typical prokaryotic signal peptide containing four positively charged residues followed by a hydrophobic domain (residues 17–34) and ending at a bacterial signal peptide cleavage site (between residues 37 and 38). Analogous signal peptides were found in the AprF, PrtF and TolC outer-membrane factors in related export systems (Letoffé *et al.*, 1990; Duong *et al.*, 1992).

Mutagenesis of *prsD*

To construct a *prsD* mutant, a *Bam*HI kanamycin resistance cassette isolated from pSUP2021 (Simon *et al.*, 1983) was inserted *in vitro* into the *Bam*HI site of *prsD*

on pJ6 (Fig. 1). Transformants were tested for the correct constructs and inserts were introduced into the genome of the wild-type strain *R. l. trifolii* TA1 by marker exchange, resulting in the *prsD* mutant TA1.34. The TA1.34 *prsD* mutant formed normal mucoid colonies resulting from the production of exopolysaccharide in the same amount as the wild-type strain. The number of nodules per clover plant was similar as in the inoculation with strain TA1. However, the nodules formed on *Trifolium pratense* did not fix nitrogen, as determined by an acetylene reduction test for nitrogenase activity.

DISCUSSION

In this study we report the identification of three new *R. l. trifolii* TA1 genes, designated *prsD*, *prsE* and *orf3*. *prsD* and *prsE* are homologous to a superfamily of ATP-binding proteins and are probably involved in transport. In addition to homology with *P. aeruginosa* *aprDE* genes (Duong *et al.*, 1992) and *Erw. chrysanthemi* *prtDE* genes (Letoffé *et al.*, 1990), we also found significant homology to the secretion guard for the *Serratia marcescens* lipase *lipBC* genes (Akatsuka *et al.*, 1995) and metalloprotease *hasDE* genes (Letoffé *et al.*, 1993). These data suggest that the exported substrate could be an extracellular protease. However, we could not detect extracellular proteolytic activity in *R. l. trifolii* TA1. Hydrolytic enzymes that cleave glycosidic bonds in plant cell walls, such as pectinases, cellulases and hemicellulases, have been found in other wild-type strains of *R. l. trifolii* but only at low levels of activity (Mateos *et al.*, 1992; Jimenez-Zurdo *et al.*, 1996). We did not find such extracellular enzymes in TA1.

Although the genes involved in the synthesis and secretion of the extracellular proteins are usually clustered, the lipase and metalloprotease genes are not linked to *lipBCD* in *S. marcescens* but are linked to genes encoding products homologous to GDP-mannose pyrophosphorylase, an enzyme involved in the biosynthesis of lipopolysaccharide in *Salmonella typhimurium* and *E. coli* (Akatsuka *et al.*, 1995). The structure of LPS plays an important role in the localization of secretory components such as TolC, PrtF and even PrtE in the outer membrane (Wandersman & Letoffé, 1993). We have also considered the involvement of an ABC transporter protein, PrsD, and accessory factor, PrsE, in the export of exopolysaccharides because of the location of the *prsDE* and *orf3* genes in the vicinity of the *exo133* mutation and the partial homology of PrsE to ExoF (22.6% identity over 199 aa). ExoF is a membrane protein involved in the first step of the biosynthesis of EPSI in *R. meliloti* (Reuber & Walker, 1993). The identification of the homology of PrsE to PrtE and of ORF3 as a putative outer-membrane protein with an N-terminal signal sequence argues against this hypothesis because at present none of the systems involved in the secretion of non-protein substrates have accessory factors like HlyD or PrtE and outer membrane factors such as TolC or PrtF (Fath & Kolter, 1993). Recently,

Becker *et al.* (1995) identified the *exsA* gene in *R. meliloti*, linked to *exoB*, encoding a protein homologous to ABC exporters that secrete non-protein substrates. ExsA is homologous to NdvA involved in the export of cyclic β -(1,2)-glucans (Stanfield *et al.*, 1988).

The *R. leguminosarum* bv. *viciae* nodulation protein NodO has been described as partially homologous to haemolysin and is secreted from *E. coli* into the growth medium by HlyBD/TolC and PrtDEF protein secretion systems. This is strong evidence that an ABC exporter is required for NodO secretion (Scheu *et al.*, 1992; Sutton *et al.*, 1994). The *Rhizobium* genes encoding the proteins required for NodO secretion are unlinked to the *nodO* gene which is located on the symbiotic plasmid. Several rhizobia, including *R. l. trifolii*, containing the cloned *nodO* gene secrete NodO into the medium (Scheu *et al.*, 1992). Our results provide further evidence of the existence of a specialized secretion system widespread among Gram-negative bacteria.

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