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EFFECTS OF *pssB* MUTATION ON SURFACE POLYSACCHARIDES AND SYMBIOTIC PHENOTYPE OF *RHIZOBIUM LEGUMINOSARUM* BV. *TRIFOLII*

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The *Rhizobium leguminosarum* bv. *trifolii pssB* gene encodes a protein with inositol monophosphatase activity (I–1-Pase) which shares sequence similarity with a family of diverse prokaryotic and eukaryotic enzymes. A *pssB::lacZ*-Gm^r mutant (Rt12A) that does not synthesize I–1-Pase exhibits a wide range of pleiotropic phenotypic alterations. These changes include establishing non-nitrogen-fixing symbiosis with clover, doubled production of exopolysaccharide (EPS) with elevated content of the high molecular weight (HMW) form, and EPS viscosity significantly higher than in the wild type strain. Moreover, changes in the LPS I form containing the O antigen were found by SDS-PAGE analysis. The absence of fucose, 2-O-methyl–6-deoxyhexose and 3-N-methyl–3,6-dideoxyhexose, and the appearance of two other 6-deoxyhexoses, confirmed changes in the LPS O polysaccharide of the *pssB* mutant. These alterations indicate that the *pssB* mutation greatly affected not only EPS processing but also biosynthesis of the O antigen of LPS. Light and electron microscopy revealed that clover nodules infected with the *pssB* mutant accumulated significantly increased quantities of starch throughout the nodule and overproduced empty membranous structures, suggesting a defect in bacterial release into plant cells.

Key words: Exopolysaccharide, lipopolysaccharide, inositol monophosphatase, PssB protein, *Rhizobium leguminosarum*.

INTRODUCTION

Azorhizobium, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* strains, collectively called rhizobia, are capable of inducing the formation of nodules on roots or stems of leguminous host plants (Perret et al., 2000). Establishment of symbiosis between a rhizobium and its host plant is a complex process involving the exchange of a series of signals between the plant and bacteria (Spaink, 2000). In response to plant-excreted flavonoids, rhizobia produce lipooligosaccharides called Nod factors. Although it is clear that Nod factors allow rhizobia to penetrate the plant root, other carbohydrates are required for infection thread development and nodule formation (Spaink, 2000). Among these, cell surface polysaccharides such as exopolysaccharides (EPS), lipopolysaccharides (LPS) and K-antigens are believed to be involved in infection thread initiation and nodule invasion (Becker and Pühler, 1998; Fraysse et al., 2003). Acidic EPS plays a crucial role in establishment of effective symbiosis between *R. leguminosarum* and the host plant, forming indeterminate nodules (Rolfe et al., 1996). EPS produced by *R. leguminosarum* is a polymer of repeating subunits of five glucoses, two glucuronic acids and one galactose with acetyl, pyruvyl, and 3-hydroxybutanoyl modifications (Philip-Hollingsworth et al., 1989). Assembly of repeating units at the undecaprenyl phosphate (IP) carrier requires several specific glycosyl transferases encoded by *pssA*, *pssDE*, *pssC*, *pssGHI* and other as yet unidentified genes of *R. leguminosarum* (Janczarek and Skorupska, 2004; Król et al., 1998; van Workum et al., 1997). The polymerized EPS is subsequently translocated outside the cell. In *R. leguminosarum* bv. *trifolii*, *pssTNOP* and *pssL* genes encode proteins which might constitute polymerization/translocation machinery (for review see Skorupska et al., 2006). Two different size classes of EPS polymer

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can be distinguished: high molecular weight molecules (HMW) consisting of thousands of saccharide units, and low molecular weight molecules (LMW) consisting of 8–40 saccharide units (Becker at al., 1995).

In *R. leguminosarum*, the *pssA* gene encodes the first IP-glucosyltransferase initiating the synthesis of octasaccharide subunits of EPS by transfer of glucose–1-phosphate from UDP-glucose to IP-carrier. Upstream of *pssA* is the *pssB* gene that encodes a protein homologous to members of the prokaryotic and eukaryotic family of inositol monophosphatases (I–1-Pase) hydrolyzing *m*-inositol–1 (or 4)-phosphate to release *m*-inositol and Pi (Janczarek et al., 1999). A mutant strain Rt12A with *pssB* inactivated by insertion of the *lacZ*-Gm^r cassette displayed increased overall production of EPS versus the wild type RtTA1. The *pssB* gene was essential for symbiotic nitrogen fixation, as the *pssB* mutant initiates ineffective nodules on clover (Janczarek et al., 1999; Janczarek and Skorupska, 2001).

Lipopolysaccharides are the major constituent of the outer membrane, composed of three entities: lipid A, the core oligosaccharide, and the O antigen consisting of repeating oligosaccharide units. Mutations that affect the presence, amount and length of the O chains result in nodulation defects, ranging from early blocks in infection thread formation to disturbance of bacterial release from infection threads (Forsberg et al., 2000; Fraysse et al., 2003; Noel et al., 2004).

This work examined the pleiotropic effects of *pssB* mutation on the physicochemical properties, structure and distribution of low and high molecular weight EPS forms in the mutant Rt12A. Besides EPS changes, alterations in the LPS PAGE-banding pattern and the O antigen sugar composition of *pssB* mutant were found. Defective symbiosis of the mutant strain Rt12A was observed.

MATERIALS AND METHODS

BACTERIAL STRAINS AND GROWTH CONDITIONS

Rhizobium leguminosarum bv. *trifolii* wild-type strain TA1 (RtTA1) and its *pssB*::*lacZ*-Gm^r mutant derivative strain Rt12A were described earlier (Janczarek et al., 1999). *R. leguminosarum* strains were grown in 79CA complete medium with 1% mannitol at 28°C (Vincent, 1970).

BACITRACIN SENSITIVITY ASSAY

The antimicrobial activity of bacitracin was determined by dilution. Approximately 10^6 ml^{-1} CFU of each *R. leguminosarum* bv. *trifolii* strain was inoculated into serial dilutions of bacitracin (2 to 100 U ml^{-1}) in 79CA medium. After incubation for 24 h at 28°C, OD_{550} was determined. The MIC was defined as the lowest concentration of bacitracin that completely inhibited visible bacterial growth.

EPS EXTRACTION AND CHROMATOGRAPHY

For EPS isolation, *R. leguminosarum* bv. *trifolii* strains were grown for 4 days at 28°C in 79CA medium. After centrifugation, EPS was precipitated from culture supernatant with 3 vol of cold ethanol. The precipitate was dried, redissolved in distilled water, dialyzed and lyophilized. EPS production was quantified by the anthrone method from bacteria grown on plates to eliminate contamination with sugars from the liquid media, as described by Gao et al. (2001). To estimate the ratio of HMW to LMW forms, total EPS was isolated from culture supernatants by Bio-Gel A–5m (BioRad) chromatography as described by Becker et al. (1995).

VISCOSITY MEASUREMENTS

Relative viscosity of bacterial suspensions was determined by measuring time required for them to pass through a 3.0 mm × 50 cm column at 25°C. The viscosity of 0.005% EPS water solutions was measured using a CVO 50 high-precision rotating rheometer (Bohlin Instruments) at 25°C. The presented values are averages of three independent measurements. To measure viscosity of EPS a number of water solutions varying in volume and containing 0.005% EPS were prepared. The obtained solutions were incubated for 12 h at 25°C, and then the viscosities (η) of the solutions were measured.

LPS ISOLATION AND ANALYSIS

LPSs were isolated by the hot phenol-water method, dialyzed, lyophilized, and purified by affinity chromatography (Polymyxin-agarose, Sigma) using a method described by Kannenberg and Carlson (2001). The LPS preparations were analyzed by Tricine sodium dodecyl sulphate-polyacrylamide electrophoresis (SDS-PAGE) with 12.5% gel and visualized by silver staining (Lesse et al., 1990). Alditol acetates were prepared by LPS hydrolysis (121°C, 2 h) in 2 M trifluoroacetic acid (TFA) followed by reduction with sodium borodeuteride and acetylation. To confirm the presence of uronic acids, methanolysis and carboxyl reduction with sodium borodeuteride prior to TFA hydrolysis was performed according to Philip-Hollingsworth et al. (1989). For amino sugar analysis, the hydrolyzed samples were N-acetylated prior to their reduction with sodium borodeuteride. Alditol acetate derivatives were identified by gas liquid chromatography-mass spectrometry (GLC-MS) on a HP–5 fused silica capillary column (Supelco).

TABLE 1. Analysis of EPS of *R. leguminosarum* bv. *trifolii*

Strain	Relative culture viscosity	Viscosity of EPS solution (η) mPa·s	Ratio of HMW:LMW (%)	Ratio of EPS repeating units/reducing ends [a]	Molar equivalents of noncarbohydrate residues/repeating units [b]		
					pyruvyl	acetyl	3-hydroxybutanoyl
RtTA1	1.0	0.871	50:50	77	1.57	0.63	0.1
Rt12A	2.1	1.263	57:43	397	1.88	0.76	0.2

[a] Values were obtained by dividing the molar concentration of the repeating units (assuming that it is one-eighth of the total sugar concentration estimated in EPS precipitated by 3 volumes of ethanol) by the measured molar concentration of reducing sugars.
[b] Values were obtained from ^{1}H NMR spectrum by comparing the integrals for methyl protons of pyruvate (δ 1.32 and 1.37) and acetate (δ 2.02 - 2.26) with that of α anomeric proton of the branching glucose residue (δ 5.38).

NMR ANALYSIS

For ^{1}H-NMR analysis, 8 mg EPS obtained by precipitation with 3 vol of ethanol from culture supernatants were dissolved in 1.0 ml D_2O (99.98 atom%D, Armar Chemicals), lyophilized, and redissolved in 0.5 ml D_2O. This step was repeated three times. ^{1}H-NMR spectra were recorded on a Bruker WM–300 spectrometer at 341 K using trimethylsilane as external standard.

PLANT GROWTH AND STARCH ANALYSIS

Clover seedlings (*Trifolium pratense* cv. Ulka) were inoculated with RtTA1 or Rt12A (Janczarek et al., 1999). To determine starch content, the nodules were harvested after 5 weeks of growth, frozen in liquid nitrogen and stored at –70°C. Frozen nodules (100–150 mg) were extracted for 15 min at 80°C. The extract was centrifuged and the precipitate was reextracted twice with ethanol. After the last centrifugation the precipitate was saved for starch determinations (Nielsen et al., 1991). The supernatants were saved for water-soluble carbohydrate determinations (Lloyd and Whelan, 1969). The undissolved plant material recovered after ethanol extraction was boiled in 0.1 M KOH for 30 min, then pH was brought to 6.9, and the gelatinized starch was treated with α-amylase from *Aspergillus oryzae* (300 U ml^{-1}, SERVA) for 40 min at 22°C (pH 6.9). Then the pH was brought to 5.0 and the sample was treated with amyloglucosidase (420 U ml^{-1}, SERVA) for 18 h at 50°C. The liberated glucose was determined enzymatically using glucose oxidase and peroxidase by enzyme-linked oxidation of o-dianizidine and monitored spectrophotometrically at 525 nm (Lloyd and Whelan, 1969).

LIGHT AND ELECTRON MICROSCOPY

Clover (*Trifolium pratense* L. cv. Ulka) nodules were harvested and prepared for light and electron microscopy as previously described by Król et al. (1998).

RESULTS

EPS ANALYSIS OF THE WILD TYPE STRAIN RTTA1 AND ITS *PSSB* MUTANT

R. leguminosarum bv. *trifolii* mutant strain Rt12A was isolated by homologous recombination of *pssB-lacZ*-Gm^r with the wild type *pssB* gene (Janczarek et al., 1999). Southern hybridization of total DNA of the Rt12A mutant with the *lacZ-Gm^r* cassette as probe showed a single hybridization band (data not shown). The Rt12A formed very mucoid, translucent colonies when grown on 79CA medium. The level of EPS production in the *pssB* mutant was twice that of the wild type strain RtTA1. To further study the effect of the *pssB* mutation on polysaccharide synthesis, the physical and chemical properties of EPS produced by strain Rt12A were analyzed (Tab. 1). Measurements of culture viscosities indicated a twofold increase over RtTA1 for Rt12A (Tab. 1). Further, the viscosities of 0.005% (w/v) water solution of EPS precipitated with ethanol from culture supernatants were measured. The viscosity [η] of EPS produced by Rt12A was 1.45 times higher than that of the wild type strain RtTA1 (Tab. 1). The difference in viscosity probably was related to the higher polymerization of EPS produced by the *pssB* mutant.

To determine the molecular weight distribution in EPS produced by the *pssB* mutant, culture supernatants were subjected to gel permeation chromatography on a Bio-Gel A5-m column (Fig. 1). Total Rt12A EPS was separated into the HMW fraction which eluted as two poorly resolved peaks representing a molecular weight of 2 to 5 MDa, and the LMW fraction which eluted as a broad peak in the range of 10 kDa (Fig. 1). The ratio of HMW to LMW EPS was found to be 57:43% for Rt12A and 50:50% for RtTA1, indicating the greater contribution of the more polymerized fraction of EPS produced by the *pssB* mutant. To confirm that the HMW and LMW fractions of both strains represented EPS, peak samples from Bio-Gel A5m were analyzed for monosaccharide composition. The ratio of glucose to glucuronic acid galactose was found to be ~5:2:1, characteristic for acidic EPS of *R. leguminosarum*.

To further characterize the degree of EPS polymerization of Rt12A and RtTA1, the ratio of the number of EPS repeating units to the number of reducing sugars reflecting the numbers of free reducing ends was calculated according to Finnie et al. (1998). As shown in Table 1, the ratio of EPS repeating units to reducing ends in the *pssB* mutant was found to be more than 5 times that estimated for the EPS of strain RtTA1.

NMR SPECTROSCOPY OF EPS OF RTTA1 AND RT12A

The degree of noncarbohydrate glycosyl substitution in nonfractionated EPS precipitated with 3 vol of ethanol from RtTA1 and Rt12A culture supernatants was determined (Tab. 1). ^{1}H-NMR spectra contained signals due to CH_3 of pyruvic acetal (δ 1.56 – 1.57), acetyl (δ 2.21, 2.26 and 2.30) and very weak signals for CH_3 (δ 1.20) and CH_2 (δ 2.62) indicating the presence of residual amounts of 3-hydroxybutanoyl groups. Proton resonances in the range of δ 3.0 – 5.4 were assigned to glycosyl components. The ratio estimated upon integration of signals corresponding to the noncarbohydrate substituents in relation to the signal due to the C–1 proton from α-branched glucose residue (δ 5.38) indicated a negligible increase in the amount of acetyl, pyruvate and 3-hydroxybutanoyl substituents in Rt12A.

ANTIBIOTIC SUSCEPTIBILITY

The Rt12A and RtTA1 strains substantially differed in sensitivity to bacitracin, an antibiotic affecting the cell envelope (MIC value 10 U ml^{-1} and 70 U ml^{-1}, respectively); their susceptibility to other antibiotics acting on the cell envelope (penicillin G, ampicillin, vancomycin, cephalosporins, polymyxin B) was comparable.

SDS-PAGE ANALYSIS

To investigate whether the *pssB* mutation affects LPS synthesis, LPSs from RtTA1 and Rt12A strains were analyzed by SDS-PAGE (Fig. 2). The LPS profiles of *R. leguminosarum* bv. *trifolii* strains were compared with the well-defined LPS of *R. etli* CE3 which separates into two bands; fast-migrating LPS II representing the core oligosaccharide and lipid A, and slow-migrating LPS I which carries the O antigen (Noel et al., 2000; Fig. 2, lane 1). LPS of strain RtTA1 exhibited two intensive bands corresponding to LPS I and LPS II (Fig. 2, lane 2). The appearance of two faintly stained bands in the upper gel region indicated the presence of LPS species containing more polymerized O chains. LPS of the *pssB* mutant showed two major well-separated bands corresponding to LPS I and LPS II. A number of almost equally intense bands were also observed in the region between these LPS species. The PAGE-banding profile suggested higher heterogeneity of LPS of the *pssB* mutant.

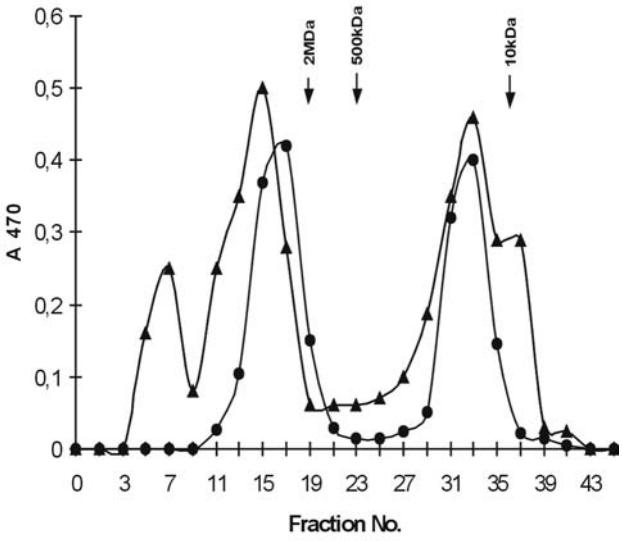


Fig. 1. Gel filtration chromatography of EPS produced by the wild type strain *R. leguminosarum* bv. *trifolii* RtTA1 (●) and the *pssB* mutant Rt12A (▲). EPS was fractionated on a Bio-Gel A5m column as described in the text. The elution of the dextran molecular mass standards (2 MDa, 500 and 10 kDa) is indicated by arrows.

LPS SUGAR COMPOSITION

The above results suggested that the differences in gel migration pattern between LPS from wild type and *pssB* mutant could be the result of changes in the O chain rather than the core region of Rt12A LPS. In order to assign the changes, the glycosyl compositions of LPSs from RtTA1 and Rt12A strains were examined (Tab. 2). The major differences between the LPSs of mutant and parent were the complete lack of fucose, 2-O-methyl–6-deoxyhexose and 3-N-methyl–3,6-dideoxyhexose, and the appearance of 6-deoxytalose and rhamnose in the LPS of the mutant. All the above sugars were detected as O antigen components, that is, occurring in the high molecular weight fractions obtained during separation of the RtTA1 degraded polysaccharide on Sephadex G–50 (data not shown).

SYMBIOTIC PHENOTYPE OF *PSSB* MUTANT

The Rt12A mutant strain induced nodules on clover in which bacteroids were unable to fix nitrogen, indicating that PssB protein is essential for effective symbiosis. The nodules were small and white, and appeared at 14–16 days after inoculation, whereas nodules induced by the parental RtTA1 strain appeared one week after inoculation (Janczarek et al., 1999).

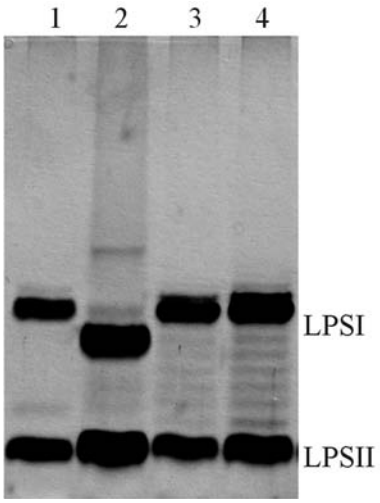


Fig. 2. Analysis of the LPS of the wild type strain *R. leguminosarum* bv. *trifolii* RtTA1 and Rt12A *pssB* mutant by silver-stained SDS-PAGE. Lane 1, reference LPS of *R. etli* strain CE3; lane 2, RtTA1; lanes 3, 4, Rt12A.

TABLE 2. Sugar composition of purified LPSs

Sugar	LPS from *R. leguminosarum* bv. *trifolii*[a]	
	RtTA1	Rt12A
2-O-Methyl-6-deoxyhexose	3.7	0
6-deoxytalose	0	9.5
Rhamnose	0	12.0
Fucose	6.9	0
Quinovosamine	3.2	1.5
3-N-Methyl-3,6-dideoxyhexose	11.3	0
Mannose	9.3	4.3
Glucose	18.2	24.2
Glucuronic acid	9.2	5.9
Galactose	7.1	12.2
Galacturonic acid	17.7	24.6
Glucosamine	3.9	1.8
Heptose	5.5	1.0
3-Deoxy-D-*manno* 2-octulosonic acid	4.0	3.0

[a]Expressed as area percentages

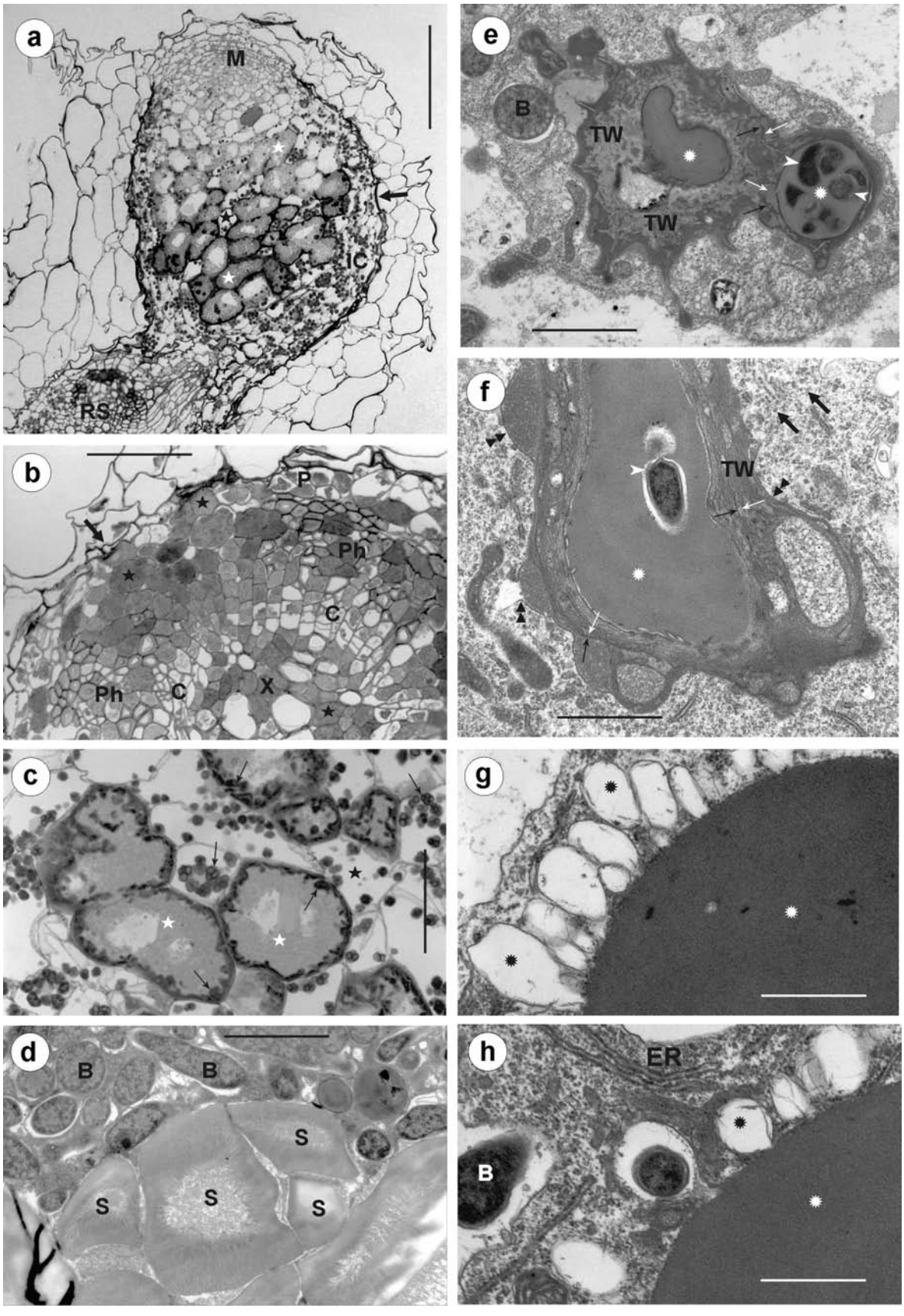
Light microscopy showed that Rt12A nodules contained fewer infected cells and that abundant starch grains accumulated throughout the nodules (Fig. 3a). The starch grains were accumulated in the infected and uninfected nodule cells, in the nodule inner cortex parenchyma, and even in the parenchymatous cells of root stele (Fig. 3a,b). In the infected cells of mutant nodules, starch-containing amyloplasts were situated parietally along the whole cell wall (Fig. 3c,d), while in the RtTA1 nodules they were smaller and usually close to the intercellular spaces (data not shown).

The structure of nodules initiated by the Rt12A mutant was examined by transmission electron microscopy (Fig. 3d-h). The ultrastructure of the infection thread wall significantly differed from that of the wild type: it contained discontinuous layers of strongly osmiophilic material, and between the layers a more homogenous and less osmiophilic substance was deposited in such a manner that the infection thread wall surface was irregular, forming protuberances (Fig. 3e,f). The bacteria embedded in the thread matrix were few and were surrounded by a very narrow electron-light zone (Fig. 3e,f). The process of bacterial release was disturbed, and frequently the plasma membrane surrounding unwalled tips of infection threads produced many vesicles devoid of any visible content (Fig. 3g,h). Similar vesicles were seen in the cytoplasm of newly infected cells, and their transfer to vacuoles was observed. The bacteria occasionally loaded to the vesicles were also eliminated to vacuoles (Fig. 3h).

To gain information on the extent of starch accumulation in the nodules of clover infected with Rt12A and RtTA1 strains, the levels of soluble and insoluble carbohydrates in the isolated nodules were determined. As expected from light and electron micrographs of nodule sections, clover nodules infected with Rt12A accumulated more starch (244 mg g^{-1} dry weight of nodules) than those of the RtTA1 strain (190 mg g^{-1}). The level of soluble carbohydrates in Rt12A and RtTA1 nodules was similar, 80 and 86 mg g^{-1}, respectively.

DISCUSSION

Disruption of the *pssB* gene encoding inositol–1-phosphatase (I–1-Pase) affects EPS synthesis by increasing the total amount of EPS, increasing the HMW form at the expense of the LMW fraction, and increasing the viscosity of the purified EPS (Fig. 1, Tab. 1). Significant alteration of the PAGE banding pattern of LPS, together with a remarkable difference between the O antigen glycosyl composition of *R. leguminosarum* Rt12A and of the wild type strain RtTA1, was also demonstrated (Fig. 2, Tab. 2). A defect of symbiosis of the *pssB* mutant was established as an inability to fix nitrogen in symbiosis with clover (Janczarek and Skorupska, 2001). Clover plants infected with the mutant strain accumulated an enormous amount of starch in the nodules and roots. The amount of starch in Rt12A nodules was 28% more than in RtTA1 nodules. This remarkable increase implies that bacteria or bacteroids unable to synthesize PssB protein and to fix nitrogen require much less carbon from the plant. Such a reduction in carbon demand from the plant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might lead to increased starch accumulation (Lodwig et al., 2005). The next striking finding in Rt12A induced nodules was overproduction of empty membranous structures, suggesting a defect in bacterial release into plant cells. This might be interpreted as disturbed plasma membrane synthesis. Overproduction of membrane structures not associated with symbiosome formation was never observed in wild type nodules (Łotocka et al., 1997).

I-1-Pases are ubiquitous in a wide range of organisms; pro- and eukaryotic and several homologous proteins can be found by searching the protein database of bacteria with sequenced genomes (Chen and Roberts, 2000). *pssB* homologues such as *psi53* in *S. meliloti* strain 104A14 (Summers et al., 1998) and *suhB* in strains 1021 and 2011 have been found (Krol and Becker, 2004). The degree of sequence similarity between particular homologous proteins with I–1-Pase activity is low, in the range of 25–30% identity. PssB of *R. leguminosarum* bv. *trifolii* TA1 shows only 25.8% identity with SuhB of *E. coli* and 28.6% with SuhB of *S. meliloti*.

In *S. meliloti* strains, *PhoB*-dependent phosphate-starvation-inducible *psi53* and *suhB* gene expression was found by global transcription analysis (Krol and Becker, 2004). In contrast, *pssB* transcription and EPS production in *R. leguminosarum* bv. *trifolii* increased in phosphate-sufficient medium (Janczarek and Skorupska, 2004). The role of PssB in overall rhizobial metabolism is not resolved, but it has been suggested that it may function in mobilization of free phosphate (Krol and Becker, 2004) rather than in the synthesis of inositol-containing lipids. In rhizobia, inositol compounds are undetected or present in minute amounts.

The present work investigated the role of PssB in EPS/LPS synthesis. The most striking difference between EPS produced by the *pssB* mutant and wild type EPS was increased viscosity and a higher ratio of total versus reducing sugars. There was also a higher contribution of the HMW at the expense of the LMW form. These data indicate that EPS processing is affected by the *pssB* mutation. Increased viscosity of EPS produced by the *R. leguminosarum* bv. *viciae* mutant in the *plyB* gene encoding a secreted polysaccharide lyase has been described (Finnie et al., 1998).

The sugar composition of the HMW and LMW EPS fractions in the wild type and mutant was identical, but the amount of pyruvyl, acetyl and 3-hydroxybutanoyl groups was somewhat greater in mutant than in wild type EPS (Tab. 1). According to Canter Cremers et al. (1991), the level of ester substitutions [O-acetyl and O-(3-hydroxybutanoyl) groups] does not influence nodulation ability, but the degree of acetylation is known to affect the rheological properties of EPS.

The complexity of the *pssB* mutation was further demonstrated as changes in the LPS PAGE-banding pattern, and as significant alterations in glycosyl composition (Tab. 2). Interpretation of changes in the sugar composition of LPS is complicated. The lack of fucose, 2-O-methyl–6-deoxyhexose and 3-N-methyl-dideoxyhexose and the appearance of 6-deoxytalose and rhamnose indicates changes in the O antigen polysaccharide. We suggest that the *pssB* mutation influences the biosynthetic pathway of 6-deoxyhexoses or switches on an alternative pathway of O antigen biosynthesis. Broughton et al. (2006) demonstrated that there are at least two regulatory, flavonoid-inducible pathways governing production of the rhamnan O antigen in *Rhizobium* sp. NGR234. Noel et al. (2004) found that the CE395 mutant of *R. etli* lacking 2-O-methylfucose in the O antigen is affected in symbiosis with *Phaseolus vulgaris* and forms pseudonodules. The alterations in the size and sugar composition of the O antigen displayed by the *pssB* mutant seem to contribute to the ineffectiveness of nodules formed by Rt12A.

The *pssB* mutant displayed enhanced susceptibility to bacitracin. The primary mode of action of bacitracin is interference with bacterial cell wall synthesis through inhibition of dephosphorylation of the peptidoglycan carrier C_{55}-isoprenyl pyrophosphate. After translocation of sugar-peptide units, the C_{55}-isoprenyl pyrophosphate (IPP) is detached and dephosphorylated to IP by a membrane-bound pyrophosphatase, thus recycling IP. This reaction of dephosphorylation, which is the site of action of bac-

Fig. 3. Light (**a-c**) and electron microscopy (**d–h**) of nodule induced by *R. leguminosarum* bv. *trifolii* Rt12A. (**a**) Longitudinal section of 2-week-old clover nodule. M – nodule meristem; IC – nodule inner cortex; RS – root stele; white asterisks – infected plant cells; black asterisks – uninfected plant cells; black arrow – nodule endodermis. Bar = 100 μm, (**b**) Transverse section of clover root stele. P – parenchymatous cells of pericycle; Ph – phloem; X – xylem; C – cambium; black asterisks – accumulated starch. Bar = 70 μm, (**c**) Nodule cells infected with bacteroids, white asterisks – infected cells; black asterisk – uninfected cell; black arrows – starch grains. Bar = 55 μm, (**d**) Ultrastructure of an infected plant cell; S – starch grains in direct contact with the cytoplasm after rupture of amyloplast envelope; B – bacteroid. Bar = 6.4 μm, (**e,f**) Ultrastructure of an infection thread; TW – infection thread wall; thin black arrows – electron-dense wall layers; white arrows – deposits of homogenous material; thick black arrows – region of cytoplasm rich in transport vesicles; rosettes – thread matrix; arrowheads – bacteria; double arrowheads – plasma membrane surrounding the infection thread. Bars in (e) = 2.4 μm, in (f) = 1.4 μm, (**g,h**) Unwalled tips of infection thread with vesicles – black rosettes; ER – endoplasmic reticulum. Bars in (g, h) = 0.9 μm.

itracin, is catalyzed by membrane-bound phosphatase (Storm and Strominger, 1973). The lipid carrier is responsible for moving the peptidoglycan, the O-specific repeating units of LPS and EPS precursors through the cytoplasmic membrane to the cell wall (Ghachi et al., 2004). The observation that the Rt12A strain is sensitive to bacitracin, unlike the wild type strain RtTA1, might suggest that IP carrier availability is limited due to increased exopolysaccharide synthesis and/or lack of I–1-Pase activity.

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REFERENCES

Becker A, and Pühler A. 1998. Production of exopolysaccharides. In: Spaink HP, Kondorosi A, Hooykaas PJJ [eds], *The Rhizobiaceae. Molecular biology of model plant-associated bacteria*, 97–118. Kluwer Academic Publishers, Dordrecht.

Becker A, Niehaus K, and Pühler A. 1995. Low molecular weight succinoglycan is predominantly produced by *Rhizobium meliloti* strains carrying a mutated ExoP protein characterized by a periplasmic N-terminal and a missing C-terminal domain. *Molecular Microbiology* 16: 191–203.

Broughton W J, Hanin M, Relic B, Kopcińska J, Golinowski W, Simsek S, Ojanen-Reuhs T, Reuhs B, Marie C, Kobayashi H, Bordogna B, Le Quéré A, Jabbouri S, Fellay R, Peret X, and Deakin WJ. 2006. Flavonoid-inducible modifications to rhamnan O antigens are necessary for *Rhizobium* sp. strain NGR234-legume symbioses. *Journal of Bacteriology* 188: 2126–2133.

Canter Cremers HCJ, Batley M, Redmond JW, Wijfjess AHM, Lugtenberg BJJ, and Wijffelmans CA. 1991. Distribution of O-acetyl groups in the exopolysaccharide synthesized by *Rhizobium leguminosarum* strains is not determined by the Sym plasmid. *Journal of Biological Chemistry* 266: 9556–9564.

Chen L, and Roberts M. 2000. Overexpression, purification, and analysis of complementation behaviour of *E. coli* SuhB protein: comparison with bacterial and archeal inositol monophosphatases. *Biochemistry* 39: 4145–4153.

Finnie C, Zorreguietta A, Hartley NM, and Downie JA. 1998. Characterization of *Rhizobium leguminosarum* exopolysaccharide glycanases that are secreted via type I exporter and have a novel heptapeptide repeat motif. *Journal of Bacteriology* 180: 1691–1699.

Forsberg LS, Bhat UR, and Carlson RW. 2000. Structural characterization of the O-antigenic polysaccharide of the lipopolysaccharide from *Rhizobium etli* strain CE3. *Journal of Biological Chemistry* 275: 18851–18863.

Fraysse N, Couderc F, and Poinsot V. 2003. Surface polysaccharide involvement in establishing the rhizobium-legume symbiosis. *European Journal of Biochemistry* 270: 1365–1380.

Gao M, Haeze WD, Derycke R, Wolucka B, and Holsters M. 2001. Knockout of an Azorhizobial dTDP-L-rhamnose synthase affects lipopolysaccharide and extracellular polysaccharide production and disables symbiosis with *Sesbania rostrata*. *Molecular Plant-Microbe Interactions* 14: 857–866.

Ghachi ME, Bouhss A, Blanot D, and Mengin-Lecreulx D. 2004. The *bacA* gene of *Escherichia coli* encodes an undecaprenyl pyrophosphatephosphatase activity. *Journal of Biological Chemistry* 279: 30106–30113.

Janczarek M, Król J, and Skorupska A. 1999. The *pssB* gene product of *Rhizobium leguminosarum* bv. *trifolii* is homologous to a family of inositol monophosphatases. *FEMS Microbiology Letters* 73: 319–325.

Janczarek M, and Skorupska A. 2001. Characterization of *Rhizobium leguminosarum* bv. *trifolii pssB* gene product as inositol monophosphatase that influences the exopolysaccharide synthesis. *Archives of Microbiology* 175: 143–151.

Janczarek M, and Skorupska A. 2004. Regulation of *pssA* and *pssB* gene expression in *Rhizobium leguminosarum* bv. *trifolii* in response to environmental factors. *Antonie van Leeuwenhoeck Journal of Microbiology* 85: 217–227.

Kannenberg EL, and Carlson RW. 2001. Lipid A and O-chain modifications cause *Rhizobium* lipopolysaccharides to become hydrophobic during bacteroid development. *Molecular Microbiology* 39: 379–391.

Krol E, and Becker A. 2004. Global transcriptional analysis of the phosphate starvation response in *Sinorhizobium meliloti* strains 1021 and 2011. *Molecular and General Genetics* 272: 1–17.

Król J, Wielbo J, Mazur A, Kopcińska J, Łotocka B, Golinowski W, and Skorupska A. 1998. Molecular characterisation and symbiotic importance of *pssD* gene of *Rhizobium leguminosarum* bv. *trifolii* strain TA1: *pssD* mutant is affected in exopolysaccharide synthesis and endocytosis of bacteria. *Molecular Plant-Microbe Interactions* 11: 1142–1148.

Lesse AJ, Campagnari AA, Bittner WE, and Apicella MA. 1990. Increased resolution of lipopolysaccharides and lipooligosaccharides utilizing tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Journal of Immnological Methods 126: 109–117.

Lloyd JB, and Whelan WJ. 1969. Enzymic determination of glucose in the presence of maltose. *Analytical Biochemistry* 30: 467–483.

Lodwig EM, Leonard M, Marroqui S, Wheeler TR, Findlay K, Downie JA, and Poole PS. 2005. Role of polyhydroxybutyrate and glycogen as carbon storage compounds in pea and bean bacteroids. *Molecular Plant-Microbe Interactions* 18: 67–74.

Łotocka B, Kopcińska J, and Golinowski W. 1997. Morphogenesis of root nodules in white clover. 1. Effective root nodules induced by wild type *Rhizobium leguminosarum* bv. *trifolii*. *Acta Societatis Botanicorum Poloniae* 66: 273–292.

Nielsen TM, Skjørbøk HC, and Karlsen P. 1991. Carbohydrate metabolism during fruit development in

sweet pepper (*Capsicum annuum*) plants. *Plant Physiology* 82: 311–319.

Noel KD, Forsberg LS, and Carlson RW. 2000. Varying the abundance of O antigen in *Rhizobium etli* and its effect on symbiosis with *Phaseolus vulgaris*. *Journal of Bacteriology* 182: 5317–5324.

Noel KD, Box JM, and Bonne VJ. 2004. 2-O-methylation of fucosyl residues of a rhizobial lipopolysaccharide is increased in response to host exudate and is eliminated in a symbiotically defective mutant. *Applied and Environmental Microbiology* 70: 1537–1544.

Perret X, Staehelin C, and Broughton W. 2000. Molecular basis of symbiotic promiscuity. *Microbiology and Molecular Biology Reviews* 64: 180–201.

Philip-Hollingsworth S, Hollingsworth RI, and Dazzo FB. 1989. Host-range related structural features of the acidic extracellular polysaccharides of *Rhizobium trifolii* and *Rhizobium leguminosarum*. *Journal of Biological Chemistry* 264: 1461–1466.

Rolfe BG, Carlson RW, Ridge RW, Dazzo RW, Mateos FB, and Pankhurst CE. 1996. Defective infection and nodulation of clovers by exopolysaccharide mutants of *Rhizobium leguminosarum* bv. *trifolii*. *Australian Journal of Plant Physiology* 23: 285–303.

Skorupska A, Janczarek M, Marczak M, Mazur A, and Król J. 2006. Rhizobial exopolysaccharides: genetic control and symbiotic functions. *Microbial Cell Factories* 5:7.

Spaink HP. 2000. Root nodulation and infection factors produced by rhizobial bacteria. *Annual Reviews of Microbiology* 54: 257–288.

Storm DR, and Strominger JL. 1973. Complex formation between bacitracin peptides and isoprenyl pyrophosphates. The specificity of lipid-peptide interactions. *Journal of Biological Chemistry* 248: 3940–3945.

Summers ML, Elkins JG, Elliott BA, and McDermott TR. 1998. Expression and regulation of phosphate stress inducible genes in *Sinorhizobium meliloti*. *Molecular Plant-Microbe Interactions* 11: 1094–1101.

Van Workum WAT, Canter Cremers HCJ, Wijfjes AHM, Van Der Kolk C, Wijffelman CA, and Kijne JW. 1997. Cloning and characterization of four genes of *Rhizobium leguminosarum* bv. *trifolii* involved in exopolysaccharide production and nodulation. *Molecular Plant-Microbe Interactions* 10: 290–301.

Vincent JM. 1970. A manual for the practical study of the root-nodule bacteria. International Biological Program Handbook no. 15. Blackwell Scientific Publications Ltd, Oxford.