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A mutation in *pssE* affects exopolysaccharide synthesis by *Rhizobium leguminosarum* bv. *trifolii,* its surface properties, and symbiosis with clover

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Abstract

Background and aims A considerable majority of the proteins involved in exopolysaccharide synthesis in *Rhizobium leguminosarum* bv. *trifolii* are encoded by *pss* genes located in a large chromosomal region named Pss-I. The aim of this work was to characterize the phenotypic and symbiotic properties of strain Rt1933, which has a mutation in *pssE.* This gene encodes an enzyme responsible for the second step of EPS unit assembly.
Methods The EPS-deficient Rt1933*pssE* strain was obtained via random mutagenesis using the mTn*5*SS*gusA*40 transposon. The mutation site in the Rt1933 genome was identified using hybridization, PCR amplification, and sequence analysis. Complementation of the *pssE* mutation was performed using biparental conjugation and a set of plasmids containing different fragments of the Pss-I region. The phenotypic properties of this mutant were established in growth kinetics experiments, as well as motility, sensitivity and hydrophobicity assays. The symbiotic proficiency of the Rt1933 strain in interaction with red clover (*Trifolium pratense*) was determined in plant tests, whereas occupation of root nodules by this mutant was investigated using light microscopy and bacteria harboring *gusA* for β-glucuronidase.

Results An *exo99* mutation in Rt1933 was identified at 3′-end of the *pssE* gene located in region Pss-I, which resulted in a lack of 16 amino acids at the C-end of PssE. This mutation totally abolished EPS synthesis in *R. leguminosarum* bv. *trifolii.* Strain Rt1933 was characterized by considerably decreased growth kinetics and motility, and an increased sensitivity to some stress factors. Also, the hydrophobicity of the mutant cells differed significantly from that of the wild-type Rt24.2 and the complemented Rt1933 cells. Moreover, the *pssE* mutant showed strong disturbances in symbiosis with clover; it induced much fewer nodules on clover roots at a later time than normal, and the mass of the plants inoculated with the mutant was significantly lower than that of the plants inoculated with the wild-type strain.
Conclusions The *pssE* gene plays a crucial role in EPS synthesis in *R. leguminosarum* bv. *trifolii,* and the presence of this polysaccharide affects the cell-surface properties of the bacterium and is required for both adaptation to stress conditions and the establishment of effective symbiosis with clover plants.

Keywords *Rhizobium leguminosarum* · *pssE* gene · Exopolysaccharide synthesis · Glycosyltransferase · Surface properties · Legume-rhizobium symbiosis · Clover

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Introduction

Rhizobium leguminosarum bv. *trifolii* is a Gram-negative soil bacterium which establishes symbiotic interactions

with clover plants (*Trifolium* spp.). Nitrogen-fixing symbiosis is a complex process which involves an exchange of many signals, among which legume flavonoids and rhizobial lipochitooligosaccharides play crucial roles (Janczarek et al. 2015c; López-Baena et al. 2016). An important part in that process is also played by acidic exopolysaccharide (EPS) synthesized in large amounts by rhizobia, which is required for the formation of nitrogen-fixing nodules on legume roots (Downie 2010). Previously, it has been established that this polymer is essential for symbiosis with host plants which form indeterminate-type nodules, such as clover, vetch, pea, and alfalfa (Rolfe et al. 1996; Chen et al. 1985; van Workum et al. 1995; Cheng and Walker 1998). Furthermore, EPS plays an important role in protecting rhizobial cells against desiccation and other stress factors occurring in soil (Jaszek et al. 2014; Fujishige et al. 2006). EPS-deficient strains of *R. leguminosarum* bvs. *trifolii* and *viciae* and *Sinorhizobium meliloti* induce nodules on compatible host plants, which are inefficient in nitrogen fixation (Ivashina et al. 1994; van Workum et al. 1997; Cheng and Walker 1998; Rolfe et al. 1996; Janczarek and Urbanik-Sypniewska 2013). However, recent data indicate that the role of EPS in legume–rhizobium symbiosis is more complex and depends on the type of host plant. For example, EPS produced by *S. fredii* strain HH103 is not required for nodulation of *Glycyrrhiza uralensis*, a host plant that also forms indeterminate-type nodules (López-Baena et al. 2016; Margaret-Oliver et al. 2012; Crespo-Rivas et al. 2015). By contrast, other data indicate that EPS is required for symbiotic interactions of some legumes which form determinate-type nodules, such as *Lotus corniculatus* and *Lotus japonicus* (Kelly et al. 2013; Muszyński et al. 2016). Kawaharada et al. (2015) have described the crucial role of EPS as a signal in early stages of symbiosis. They identified a receptor-like kinase EPR3 in the root-hair membrane of *L. japonicus* which was involved in the recognition and binding of EPS from a compatible bacterium *Mesorhizobium loti*. Moreover, a conditional requirement for EPS was found in this symbiotic model, since mutants of *M. loti* R7A strain affected in different steps of EPS synthesis showed significant differences in the effectiveness of their symbiotic interactions with *L. corniculatus* and *L. japonicus*. For example, an *exoB* mutant affected in an early stage of EPS synthesis induced nitrogen-fixing nodules on these host plants (following a delay), whereas an *exoU* mutant,

which synthesized pentasaccharide monomers, induced empty nodule-like structures on the host roots, inefficient in nitrogen fixation (Kelly et al. 2013; Muszyński et al. 2016).

In the case of *R. leguminosarum*, the structure of its EPS has been determined in detail. This polysaccharide is composed of octasaccharide subunits which contain D-glucose, D-glucuronic acid, and D-galactose residues in a molar ratio 5:2:1, and are additionally substituted with O-acetyl and pyruvyl groups (Robertsen et al. 1981; McNeil et al. 1986; Cremers et al. 1991; O'Neill et al. 1991; Breedveld et al. 1993; Philip-Hollingsworth et al. 1989). However, relatively little is known of the genetic control of EPS biosynthesis in *R. leguminosarum*. Up to now, the functions of only a few proteins participating in the five steps of EPS unit assembly have been identified experimentally. The assembly is initiated by the PssA protein which transfers glucose-1-phosphate from UDP-glucose to a C_{55}-isoprenylphosphate carrier anchored in the bacterial inner membrane (Pollock et al. 1998). Glucuronosyl-($\beta1 \rightarrow 4$)-glucosyltransferase PssDE is engaged in the second step, whereas glucuronosyl-($\beta1 \rightarrow 4$)-glucuronosyltransferase PssC in the third step of EPS unit assembly. The fourth sugar is added to the growing subunit by glucosyl-($\alpha1 \rightarrow 4$)-glucuronosyltransferase PssS (Ivashina and Ksenzenko 2012; Janczarek et al. 2015b). The PssD, PssE, PssC, and PssS proteins are encoded by genes located in a large chromosomal cluster named Pss-I (Król et al. 2007). The only exception is PssA encoded by a gene located at a long distance from this EPS synthesis region (Ivashina et al. 1994; van Workum et al. 1997). The structural and functional organization of the Pss region has been characterized in three *R. leguminosarum* strains (VF39, TA1, Rt24.2) (Sadykov et al. 1998; Król et al. 2007; Janczarek et al. 2015b). Mutations in *pssA*, *pssD*, and *pssS* genes totally abolish EPS production, whereas a mutation located in the *pssC* upstream region results in a nearly two-fold decrease in EPS biosynthesis in comparison to the wild-type strain (Ivashina et al. 1994; van Workum et al. 1997; Janczarek et al. 2015b). *pssJ* encodes a protein which is probably engaged in the last step of subunit assembly, since the *exo*344::Tn5 strain carrying a mutation in this gene has been found to synthesize only residual amounts of EPS, whose units lack a terminal D-galactose (Breedveld et al. 1993). *pssM*, which is also located in the Pss-I region, encodes a ketal pyruvate transferase responsible for one of the non-sugar modifications of EPS, i.e. pyruvylation in the subunit of the

subterminal D-glucose (Ivashina et al. 2010). A mutation in *pssM* has been observed to negatively affect symbiosis of *R. leguminosarum* bv. *viciae* with compatible host plants. So far, genes encoding proteins involved in the remaining steps of EPS unit assembly have not been identified. Proteins encoded by *pssT*, *pssN*, *pssO*, *pssP*, and *pssL* genes, which are also located in the Pss-I region, are engaged in the polymerization and export of EPS (Mazur et al. 2005; Marczak et al. 2013, 2014).

Regulation of EPS biosynthesis in *R. leguminosarum* has been shown to involve several proteins, such as PsiA, PsrA, ExoR, and RosR, and environmental factors (e.g., phosphate and nitrogen limitation, carbon source, legume root exudates) (Borthakur and Johnston 1987; Borthakur et al. 1988; Reeve et al. 1997; Janczarek and Skorupska 2011; Rachwał et al. 2015). Among these proteins, PsiA, PsrA, and ExoR participate in negative regulation of EPS synthesis, whereas RosR is a positive regulator of this process.

Two very interesting Pss proteins involved in EPS biosynthesis of *R. leguminosarum* are PssD and PssE, which, as reported by Pollock and others (Pollock et al. 1998), are required for the addition of a second sugar residue (UDP-glucuronic acid) to growing EPS repeating units. PssD and PssE are homologous to N-terminal and C-terminal regions, respectively, of glycosyltransferase SpsK of *Sphingomonas* S88, as well as to proteins Cps14F and Cps14G of *Streptococcus pneumoniae* (van Workum et al. 1997; Sadykov et al. 1998). SpsK is involved in sphingan synthesis in *Sphingomonas* S88, whereas Cps14G is responsible for the second step of CPS synthesis in *S. pneumoniae* (Kolkman et al. 1996; Yamazaki et al. 1996). Moreover, van Workum and others (1997) have identified a sequence motif in the central part of *R. leguminosarum* PssE which showed a similarity to the $QX_{13}TX_2\underline{G}X_7LX_4\underline{P}X_4PX_3DQX_4A$ consensus proposed as a UDP-binding site (Hundle et al. 1992). However, the similarity found in this motif was not high, since only three out of the nine conserved amino acids were identical (these residues are underlined).

In this study, we report that a mutation in *pssE* influences some of the physiological properties and symbiotic behavior of *R. leguminosarum* bv. *trifolii* cells. We characterize the Rt1933*pssE* mutant strain obtained via a random mutagenesis and show that the mutation in the 3′-end of this gene affects EPS synthesis, cell-surface properties and the effectiveness of its symbiosis with clover.

Materials and methods

Strains, plasmids and growth conditions

Strains, plasmids and oligonucleotide primers used in this study are listed in Table 1. *E. coli* strains were grown in LB medium at 37 °C (Bertani 1951), whereas *R. leguminosarum* bv. *trifolii* strains were cultured at 28 °C in two energy-rich media (tryptone-yeast extract [TY] and 79CA with 1% glycerol as a carbon source) and minimal M1 medium with 1% glycerol (Beringer 1974; Kowalczuk and Lorkiewicz 1979). When required, antibiotics were used at the following final concentrations: rifampicin, 40 μg ml^{-1}; kanamycin, 40 μg ml^{-1}; tetracycline, 10 μg ml^{-1}; ampicillin, 100 μg ml^{-1}; and spectinomycin, 40 μg ml^{-1}. To compare growth kinetics of Rt24.2, Rt1933, and Rt1933(pM3), the strains were cultured over 72 h in 79CA, TY, and M1 media. After 0, 24, 48, and 72 h, the optical density (OD$_{600}$) of these cultures was measured, and 100-μl aliquots were taken and their serial dilutions were placed onto agar plates, incubated at 28 °C and the colonies appearing were counted (CFU - colony-forming units). The experiment was repeated twice with three biological replicates for each strain and medium tested.

DNA methods and sequence analysis

Standard techniques were used for genomic and plasmid DNA isolation, restriction enzyme digestion, conjugation, hybridization, PCR reaction, and sequencing (Sambrook et al. 1989). For PCR amplifications, REDTaq Ready PCR Reaction Mix (Sigma-Aldrich, St. Louis, MO, USA) was used. PCR products obtained were sequenced using the BigDye terminator cycle sequencing kit and the ABI Prism 310 sequencer (Applied Biosystems, Waltham, MA, USA). Database searches were done with the BLAST and FASTA programs available from the National Center for Biotechnology Information (Bethesda, MD, USA) and the European Bioinformatic Institute (Hinxton, UK). Amino acid sequence analyses were performed using the BLASTP program (http://blast.ncbi.nlm.nih.gov/) and the CAZy database (www.cazy.org/).

Mutagenesis of the *pssE* gene

To obtain derivatives of *R. leguminosarum* bv. *trifolii* Rt24.2 defective in EPS production, a random

Table 1 Bacterial strains, plasmids and oligonucleotide primers used in this study

Strains, plasmids and primers	Relevant characteristics	Sources or reference
R. leguminosarum bv. *trifolii*		
Rt24.2	Wild type, Rif^r, Nx^r	Janczarek et al. 2015b
Rt1933	Rt24.2, *pssE99::*mTn5SS*gusA*40 (*exo99*), Sp^r	This work
Rt1933(pM3)	Rt1933 containing *pssE99::*mTn5SS*gusA*40 mutation complemented by *pssE* on pM3 plasmid, Sp^r, Km^r	This work
E. coli		
DH5α	*supE*44 Δ*lac*U169 (φ80 *lacZ*Δ M15) *hsdR*17 *recA*1*endA*1*gyrA*96 *thi*-1 *relA*1	Sambrook et al. 1989
S17–1	294, *thi*, RP4–2-Tc::Mu-Km::Tn7	Simon et al. 1983
mTn5SS*gusA*40	miniTn5 interposon with promoterless *gusA* gene, Sp^r	Wilson et al. 1995
Plasmids		
pBBR1MCS-2	cloning vector, *mob*, *lacZ*α, Km^r	Kovach et al. 1995
pARF136	pLAFR3 with 19-kb fragment of the Pss-I region complementing the *exo99* mutation	Król et al. 1998
pARF1368	pRK7813 with 5.6-kb *Bam*HI fragment of pARF136 complementing the *exo99* mutation	Król et al. 1998
pM3	pBBR1MCS-2 with the 1.4-kb *Hin*dIII-*Bam*HI fragment containing the *pssDE* genes	This work
pJBA21Tc	pMP220 containing *gusA* for β-glucuronidase	Wielbo and Skorupska 2001
Primers		
pssE9	GACAGCTACATCTCGGCACATCA	This work
prsD11	GCATTGTCGTTGTCATCGCACAT	This work
MJ-gusR2	CAGCAATTGCCCGGCTTTCTTGTAA	This work
Hind124	5′-CTCACCC<u>AAGCTT</u>TGCGCGGCGC-3′	This work
Bam113	5′-GTCAAAC<u>GGATCC</u>CCGGCAACAATTG-3′	This work

Restriction sites for *Hin*dIII and *Bam*HI enzymes are underlined

Nx^r Nalidixic acid resistance, *Rif^r* Rifampicin resistance, *Tc^r* Tetracycline resistance, *Sp^r* Spectinomycin resistane, *Km^r* Kanamycin resistance

mutagenesis of this strain was performed using S17–1 which contains the mTn5SS*gusA*40 transposon with a promoterless *gusA* gene (Wilson et al. 1995). As a result of several biparental conjugation experiments, a total of nearly 2000 colonies were obtained, among them a mutant strain characterized by a non-mucoid phenotype, named Rt1933. The localization of mTn5SS*gusA*40 in the Rt1933 genome was determined using hybridization, PCR, and sequencing analyses. Primers complementary to different *pss* genes of the Pss-I region and primer MJ-gusR2 complementary to the 3′-end of *gusA* in the mTn5SS*gusA*40 transposon were used in several PCR reactions. Only in the case of two primer pairs, pssE9/MJ-gusR2 and prsD11/MJ-gusR2, PCR products (0.5 kb- and 3.0 kb-long, respectively) were obtained. On the basis of a sequencing analysis of these products, mTn5SS*gusA*40 was identified in *pssE* of

the Pss-I region (13,600/13,601 nt, GenBank acc. no. KP067320).

Complementation of the *pssE* mutation

For complementation of the *pssE* mutation in the Rt1933 strain, pARF136, pARF1368, and pM3 plasmids containing different fragments of the Pss-I region were used. pARF136 and pARF1368 had been constructed previously by Król et al. (1998), whereas pM3 was obtained in this present study. To construct pM3, the pBBR1MCS-2 vector and a 1.4-kb fragment obtained in a PCR reaction with primers Hind124 and Bam113 complementary to the *pssD* and *pssE* genes, respectively, were used. The PCR product was digested with *Hin*dIII and *Bam*HI enzymes, cloned into the corresponding sites in pBBR1MCS-2, and the insert of the

resulting plasmid pM3 was verified by sequencing. The pARF136, pARF1368, and pM3 plasmids were introduced into *E. coli* S17–1 by transformation and subsequently into Rt1933 by biparental conjugation according to Sambrook et al. (1989).

EPS isolation and quantification

EPS isolation and quantification were performed using bacterial cultures of Rt24.2, Rt1933, and Rt1933(pM3). These strains were grown in 10 ml of 79CA for 72 h at 28 °C. The cultures after 24, 48 and 72 h of growth were centrifuged for 20 min at 14,000 rpm, and EPS was precipitated from supernatants with 4 vol. of 96% cold ethanol. After centrifugation, the EPS obtained was dissolved in water and analyzed according to Loewus (1952). The total sugar content was calculated as glucose equivalents. The experiment was repeated twice with three replicates for each strain analyzed.

Cell hydrophobicity assay

The hydrophobicity of Rt24.2, Rt1933, and Rt1933(pM3) cells was determined using a two-phase method and dodecane (Sigma-Aldrich, St. Louis, MO, USA) according to Neu and Poralla (1990) with minor modifications (Rachwał et al. 2016). Bacteria were collected from 79CA agar plates and suspended in PUM buffer (22.2 g $K_2HPO_4 \times H_2O$, 7.26 g KH_2PO_4, 1.8 g urea, 0.2 g $MgSO_4 \times 7H_2O$, 1 l water) to an optical density of ~0.5 (OD_1) at 405 nm. Then, 100 µl of dodecane was added to 200-µl aliquots of bacterial suspensions and vigorously vortexed for 2 min. After 15-min equilibration at room temperature, the lower phase of these samples was collected, and optical density was measured (OD_a) in a microplate reader (Biochrom Asys UVM 340). The degree of hydrophobicity was calculated as follows: % hydrophobicity = $100-100(OD_a/OD_1)$. The experiment was repeated three times with three replicates for each strain analyzed.

LPS profile

The LPS profiles of Rt24.2, Rt1933, and Rt1933(pM3) strains were determined as previously described (Janczarek et al. 2010). Briefly, 48-h cultures in 79CA were centrifuged, and the bacterial pellets obtained were washed twice with 0.9% NaCl to remove EPS. Then, the pellets were used to obtain LPS profiles according to Lesse et al. (1990). The samples were separated by 12.5% Tricine SDS-PAGE, and LPS was visualized by silver staining.

Motility assay

The motility of the rhizobial strains was determined as previously described (Janczarek and Rachwał 2013). For this purpose, 5-µl aliquots of bacterial suspensions of an optical density $OD_{600} = 0.2$ prepared in sterile water were stabbed into plates containing 0.3% and 0.7% 79CA, 0.3% and 0.7% TY, and 0.3% and 0.7% M1 media. The bacteria were incubated at 28 °C for 7 days, and then the migration distance from the injection site was measured. The assay was repeated three times with three replicates for each strain and each condition tested.

Assay of sensitivity to stress factors

To establish the sensitivity of the Rt24.2, Rt1933, and Rt1933(pM3) strains to sodium dodecyl sulfate, sodium deoxycholate, and ethanol, the minimal inhibitory concentrations of the tested factors were determined. To this end, 10-µl aliquots of bacterial suspensions of $OD_{600} = 0.2$ prepared in sterile water were placed on 79CA agar plates containing defined concentrations of the individual compounds (SDS: 0.05–1% *w/v*, DOC: 0.05–1% *w/v*, ethanol: 0.05–6% *v*/v). The plates were incubated for 4 days at 28 °C, and bacterial growth was established. The experiment was repeated three times with three replicates for each strain and factor tested.

Plant experiments

The symbiotic properties of strains Rt24.2, Rt1933 and Rt1933(pM3) were established using red clover (*Trifolium pratense* cv. Diana) as a host plant. Clover seedlings were transferred onto Fåhraeus agar slants (Vincent 1970), and, after 4 days, inoculated with bacterial suspensions ($OD_{600} = 0.2$, 100-µl aliquot plant^{-1}). Clover plants were subsequently grown for 4 weeks in a greenhouse under natural light supplemented with artificial light (14 h at 24 °C and 10 h at 18 °C), and root nodules were counted after each week. 28-day-old plants were harvested and weighted. The experiment was done in triplicate using 40 plants in each experiment for each strain tested.

The influence of EPS on the symbiotic behavior of the *pssE* mutant was established using clover seedlings inoculated with Rt1933 and Rt24.2 at a 1:1 ratio (live strains) or live Rt1933 and dead Rt24.2 (previously killed by heating for 10 min at 95 °C) (1:1 ratio) or live Rt1933 together with added EPS, which was isolated from Rt24.2 cultures (50 μg plant^{-1}), and individual live Rt1933 and Rt24.2 as control strains. All bacterial suspensions had an optical density $OD_{600} = 0.2$ and were used in 100-μl aliquots per seedling. Clover plants were grown for 4 weeks, and nodules were counted after each week. Then, the plants were harvested and weighed. The experiment was repeated three times using 20 plants for each strain and condition tested. The results obtained were subjected to an analysis of variance (a linear ANOVA model) with three replicates using Microsoft® Excel 2000 for Windows. *P* values 0.05 were considered significant.

In addition, nodule occupation by the bacteria tested in these conditions was established according to a method described earlier (Janczarek et al. 2009). Briefly, 12 nodules of a similar size were chosen for each condition tested. They were surface sterilized, crushed, suspended in 1 ml of 79CA, placed in serial dilutions on agar plates, and incubated at 28 °C, after which the colonies appearing were counted.

Visualization of nodule occupation using light microscopy

To determine nodule occupation by Rt1933, we used β-glucuronidase activity encoded by *gusA* present in mTn5SS*gusA*40. Rt24.2 harboring the pJBA21Tc plasmid with *gusA* and Rt1933(pM3) were used as control strains. Clover seedlings inoculated with the mutant and control strains were grown up to 4 weeks, and nodules appearing on the roots were stained for 3 h using 50 mM sodium phosphate buffer (pH 7.2) containing 50 μg ml^{-1} of 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (Janczarek and Rachwał 2013) and analyzed under a Nikon light microscope (OPTIPHOT2).

Results

Genetic characterization of mutant strain Rt1933 and complementation of a *pssE* mutation

Previously, we had performed a sequence analysis of the Pss-I region involved in EPS synthesis of

R. leguminosarum bv. *trifolii* Rt24.2 and established its genetic organization and transcriptional activity of the *pss* genes located in it (Janczarek et al. 2015b). In this study, we characterized a mutant strain named Rt1933 obtained via a random mutagenesis of the Rt24.2 strain with the use of the mTn5SS*gusA*40 transposon (Wilson et al. 1995). This mutant formed small non-mucoid colonies on 79CA agar plates, which considerably differed from those formed by the wild-type strain (data not shown). Based on hybridization, PCR amplifications with several primers complementary to different *pss* genes, and sequencing analyses, the localization of the mTn5SS*gusA*40 transposon in Rt1933, designated as the *exo99* mutation, was identified inside the Pss-I region in the *pssE* gene (a position between 13,600 and 13,601 nt, GenBank Acc. no. KP067320) (Fig. 1). In addition, the presence of one copy of mTn5SS*gusA*40 in the Rt1933 genome was confirmed using Southern hybridization and a *gusA* probe (data not shown). The *pssE* gene encodes a 179-aa long protein (a coding region from 13,112 to 13,651 nt), which shows a homology to the glycosyltransferases from the GTB-type superfamily, GT family 28 (EC 2.4, CAZy database). The mTn5SS*gusA*40 insertion in the Rt1933 genome found 50 nt upstream of the 3′-end of the *pssE* gene, resulted in a lack of 16 aa at the C-end in the encoded PssE protein.

Next, we performed a complementation analysis of the *exo99* mutation in Rt1933 using a set of plasmids: pARF136 containing a large part of the Pss-I region (i.e., genes from *pssH* to *pssN*), pARF1368 containing a 5.6-kb fragment with *pssCDEprsDE* genes, and pM3 containing a 1.4-kb fragment with *pssDE* genes and their promoter sequence (Fig. 1). In our earlier work, we had established that the *pssDE* genes were transcribed together from one common promoter (Janczarek et al. 2015b). As a result of this complementation analysis, the mucoid phenotype of the Rt1933 mutant was recovered after introduction of all of the plasmids used (pARF136, pARF1368 and pM3). This indicated that the shortest fragment of the Pss-I region present in pM3 was sufficient for complementation of the *exo99* mutation (Fig. 1). We determined the amount of EPS synthesized by the wild-type Rt24.2, Rt1933 mutant and the complemented Rt1933(pM3) strains during 72-h bacterial growth (Table 2). Both control strains, Rt24.2 and Rt1933(pM3), synthesized large amounts of EPS, the production of which increased during prolonged bacterial growth. By contrast, Rt1933 did not produce any EPS even after 72 h of
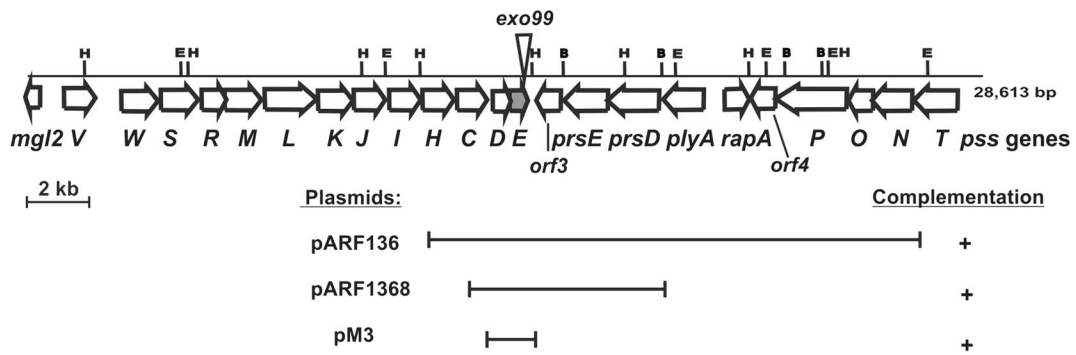
growth, indicating that the *pssE* mutation totally abolished the synthesis of this polysaccharide. These data confirm that *pssE* plays an essential role in EPS synthesis in *R. leguminosarum*.

In addition, we checked whether the *pssE* mutation affected the synthesis of another surface polysaccharide, lipopolysaccharide (LPS), in Rt1933. For this purpose, LPS profiles of Rt24.2, Rt1933 and Rt1933(pM3) were obtained and compared (Fig. 2). The analysis showed that the LPS profiles of these strains were very similar, indicating that the mutation in *pssE* did not affect the synthesis of this polysaccharide.

The *pssE* mutation affects bacterial growth, motility, and surface properties of *R. leguminosarum* bv. *trifolii*

Next, we characterized other phenotypic properties of the *pssE* mutant. First, the ability of Rt1933 to grow in

two energy-rich media (79CA and TY) and the minimal M1 medium was compared with that of the Rt24.2 and Rt1933(pM3) strains (Fig. 3). In order to eliminate the influence of EPS on this parameter, CFU (colony-forming unit) values for these strains were determined, instead of the optical density of their bacterial cultures. The growth kinetics of Rt24.2, Rt1933(pM3) and Rt1933 cells were established after 24, 48, and 72 h. Rt1933 showed slower growth in all of the tested growth media in comparison to both control strains. In 72-h

Table 2 Exopolysaccharide production by wild-type *R. leguminosarum* bv. *trifolii* Rt24.2 and its derivatives Rt1933 and Rt1933(pM3)

Strain	Total EPS (mg liter^{-1} of OD$_{600}$ = 1)[a]		
	24 h	48 h	72 h
Rt24.2 (wt)	891 ± 98[A]	1190 ± 134[A]	1287 ± 156[A]
Rt1933(*pssE*)	0 ± 0[B]	0 ± 0[B]	0 ± 0[B]
Rt1933(pM3)	912 ± 106[A]	1211 ± 126[A]	1319 ± 142[A]

[a] The given values are averages ± SD of three independent experiments with three biological replicates for each strain analyzed. The data in the column followed by different letters are significantly different ($P < 0.05$; ANOVA, post hoc Tukey's test).
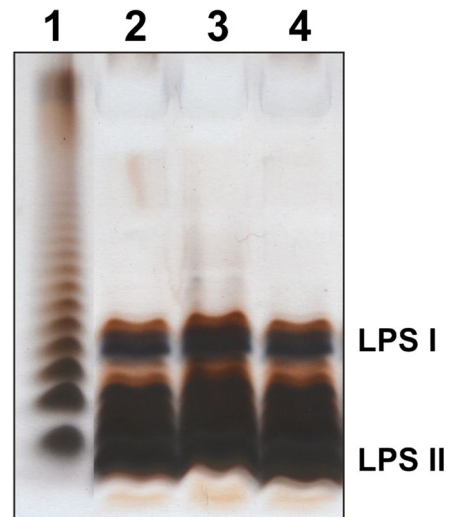

Fig. 2 Silver-stained Tricine SDS-PAGE profiles of LPS from the wild-type *R. leguminosarum* bv. *trifolii* and its derivatives. The LPSs (4 μg) were loaded into each well. Lanes: 1 – *Salmonella enterica* sv. Typhimurium (Sigma), 2 – Rt24.2, 3 – Rt1933, and 4 – Rt1933(pM3). LPS I, high-molecular-weight LPS; LPS II, low-molecular-weight LPS
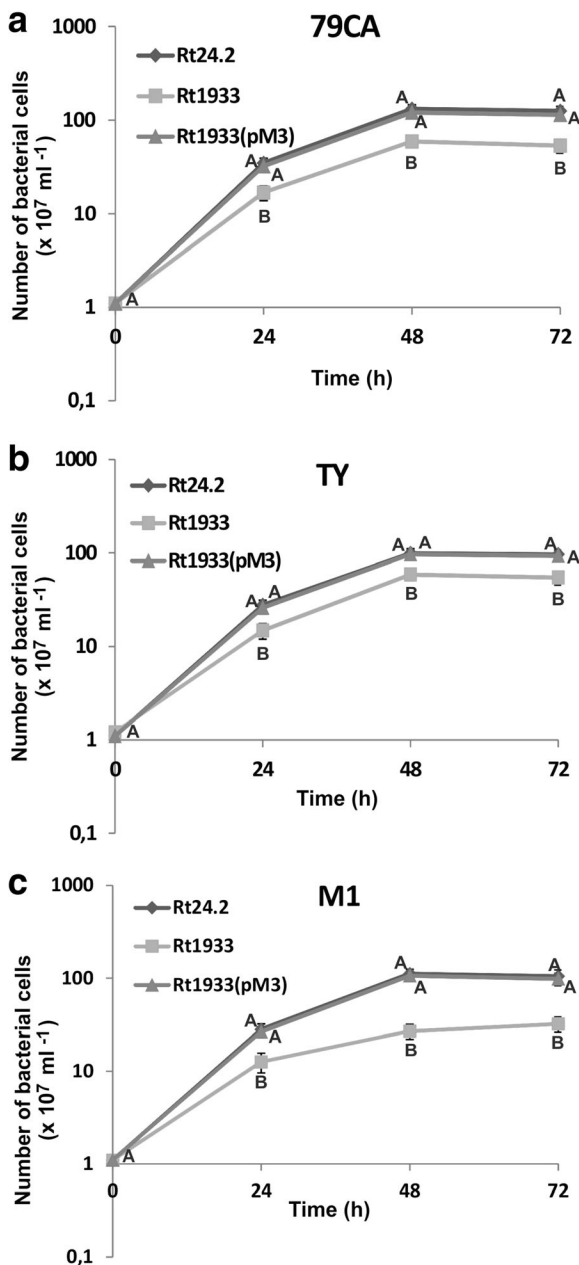
Fig. 3 The growth of *R. leguminosarum* bv. *trifolii* Rt24.2 and its Rt1933 and Rt1933(pM3) derivatives in energy-rich 79CA **a**, TY **b**, and minimal M1 medium **c**. The data are mean ± SD for three experiments. Differences statistically significant for tested strains in the particular time point are marked by different letters ($P < 0.05$; ANOVA, post hoc Tukey's test)

cultures, the growth rate observed for the *pssE* mutant was approx. two- or three-fold lower than that of the Rt24.2 and Rt1933(pM3) strains, depending on the type of medium tested. This was especially visible in the case of the minimal M1 medium.

Moreover, the motility of the *pssE* mutant and wild-type cells was determined. The ability of these bacteria to migrate was tested using 79CA, TY, and M1 media containing 0.3% and 0.7% agar (Table 3). Rt24.2 and Rt1933(pM3) showed similarly high levels of motility in both energy-rich media. The largest migration distance for these strains was found in media containing 0.3% agar. The migration distance for Rt24.2 and Rt1933(pM3) in M1 was slightly lower than in 79CA and TY. Compared to the control cells, Rt1933 cells exhibited significantly reduced motility in all the tested growth media. The largest differences in migration between the mutant Rt1933 and the Rt24.2 and Rt1933(pM3) strains were found in 0.3% M1 (~2.5-fold) and 0.3% TY (~3.3-fold).

Furthermore, the sensitivity of Rt24.2, Rt1933 and Rt1933(pM3) to stress factors such as SDS, DOC, and ethanol was established. The Rt1933 mutant was characterized by an increased sensitivity to SDS and DOC in comparison to the wild-type strain (Table 4).

Next, the hydrophobicity of *pssE* mutant cells was determined and compared with that of the wild-type cells using suspensions of bacteria collected from 79CA agar plates and a two-phase method with dodecane. In this assay, the hydrophobicity of the wild-type and the complemented *pssE* mutant strains whose cells were surrounded by a polysaccharide capsule was similar [% hydrophobicity for Rt24.2 = 16.12% ±1.82 and Rt1933(pM3) = 15.89% ±1.74, respectively]. By contrast, Rt1933 cells were characterized by a nearly 4-fold lower hydrophobicity than the cells of the Rt24.2 and Rt1933(pM3) strains [Rt1933 = 4.12% ± 1.13; $P < 0.05$, ANOVA, post hoc Tukey's test].

All these data indicate that the *pssE* mutation affected bacterial growth, motility, and surface properties of *R. leguminosarum* bv. *trifolii*.

The *pssE* mutation leads to disturbances in the symbiosis of *R. leguminosarum* bv. *trifolii* with clover

In further experiments, we investigated whether the changes observed in the phenotypic properties of the Rt1933 mutant affected its symbiotic interaction with the host plant. Red clover (*Trifolium pratense*) seedlings were inoculated with strains Rt24.2, Rt1933 and Rt1933(pM3) and grown for four weeks (Fig. 4). The control strains Rt24.2 and Rt1933(pM3) were highly effective in invading clover plants. On day 7 post

Table 3 Motility of wild-type *R. leguminosarum* bv. *trifolii* Rt24.2 and its derivatives tested in various media[a]

Strain	Motility assay (mm)[b]					
	79CA		TY		M1	
	0.3%	0.7%	0.3%	0.7%	0.3%	0.7%
Rt24.2 (wt)	42 ± 3^A	16 ± 1^A	36 ± 2^A	8 ± 1^A	25 ± 2^A	6 ± 1^A
Rt1933(*pssE*)	23 ± 2^B	6 ± 1^B	16 ± 1^B	3 ± 1^B	10 ± 1^B	3 ± 1^B
Rt1933(pM3)	41 ± 3^A	17 ± 2^A	38 ± 2^A	7 ± 1^A	27 ± 2^A	6 ± 1^A

[a] The given values are averages ± SD of three independent experiments with three biological replicates for each strain and treatment tested.

[b] The distance of bacterial migration from the injection site into agar was determined after a 7-day incubation at 25 °C. The data in the column followed by different letters are significantly different ($P < 0.05$; ANOVA, post hoc Tukey's test).

inoculation (dpi), ~30% of the plants inoculated with these bacteria had nodules on their roots, and after 21 dpi nodules were visible on all of the tested plants (100%) (Fig. 4a). By contrast, the capacity of the *pssE* mutant to infect clover roots was considerably reduced with the first nodules appearing as late as two weeks into the experiment (Fig. 4b). Moreover, only ~50% of the tested plants were infected and had nodules even after four weeks post inoculation. Apart from the delay in nodule formation, also the total number of nodules elicited on clover roots by the *pssE* mutant was essentially lower (~3-fold) than for Rt24.2 and Rt1933(pM3) (Fig. 4b). Plants inoculated with this mutant were small, had yellow leaves, and formed non-numerous small, often irregularly shaped, white nodule-like structures, whose morphology suggested they were ineffective in nitrogen fixation. This was additionally confirmed by the mass of the upper part of the clover plants inoculated with Rt1933, which was nearly 2-fold lower than the

Table 4 Sensitivity of the wild-type *R. leguminosarum* bv. *trifolii* Rt24.2 and its derivatives to various stress factors

Strain	Minimal inhibitory concentration[a]		
	SDS (% *w*/v)	DOC (% *w*/v)	Ethanol (% *v*/v)
Rt24.2 (wt)	0.025 ± 0.005^A	0.10 ± 0.005^A	5.0 ± 0.25^A
Rt1933(*pssE*)	0.010 ± 0.005^B	0.08 ± 0.005^B	4.5 ± 0.25^A
Rt1933(pM3)	0.025 ± 0.005^A	0.095 ± 0.005^A	4.75 ± 0.25^A

[a] The given values are averages ± SD of three independent experiments with three biological replicates for each strain and treatment.

The data in the column followed by different letters are significantly different ($P < 0.05$; ANOVA, post hoc Tukey's test).

mass of the plants inoculated with Rt24.2 and Rt1933(pM3), and similar to that of uninoculated plants (Fig. 4c). This was in contrast to the clover plants inoculated with Rt24.2 and Rt1933(pM3), which were tall, green, and had many pink, elongated nodules on their roots.

Next, the occupation of root nodules by the *pssE* mutant and wild-type *R. leguminosarum* cells was examined. For this experiment, Rt24.2 harboring the pJBA21Tc plasmid with *gusA* for β-glucuronidase was used. In the case of Rt1933 and Rt1933(pM3), β-glucuronidase activity was encoded by *gusA* located in the mTn5SS*gusA*40 transposon. In this experiment, we observed that both Rt24.2(pJBA21Tc) and Rt1933(pM3) occupied nodules very effectively, and these bacteria were found in both young (7-dpi) (Fig. 5a and c) as well as older (21-dpi) nodules (Fig. 5b and d). β-glucuronidase activity was detected in all zones of the 21-dpi nodules (i.e., infection zone II, interzone II–III, and nitrogen-fixing zone III). The only exception was the meristem (zone I). By contrast, occupation of clover root nodules by the Rt1933 mutant was drastically affected. Mutant cells were visible only on the surface of roots and nodules, and plant root tissues inside the nodules were not occupied by this strain (Fig. 5e–i). These observations indicated strong disturbances already in early stages of the symbiosis between the *pssE* mutant and its host plant (i.e., infection of host roots). As a consequence of these abnormalities, the nodules formed were not occupied by the microsymbiont and were unable to fix nitrogen.

Another problem that we investigated was how nodulation of clover by Rt1933 was improved by adding EPS isolated from the Rt24.2 culture and by co-inoculation with Rt24.2. Co-inoculations of host plant
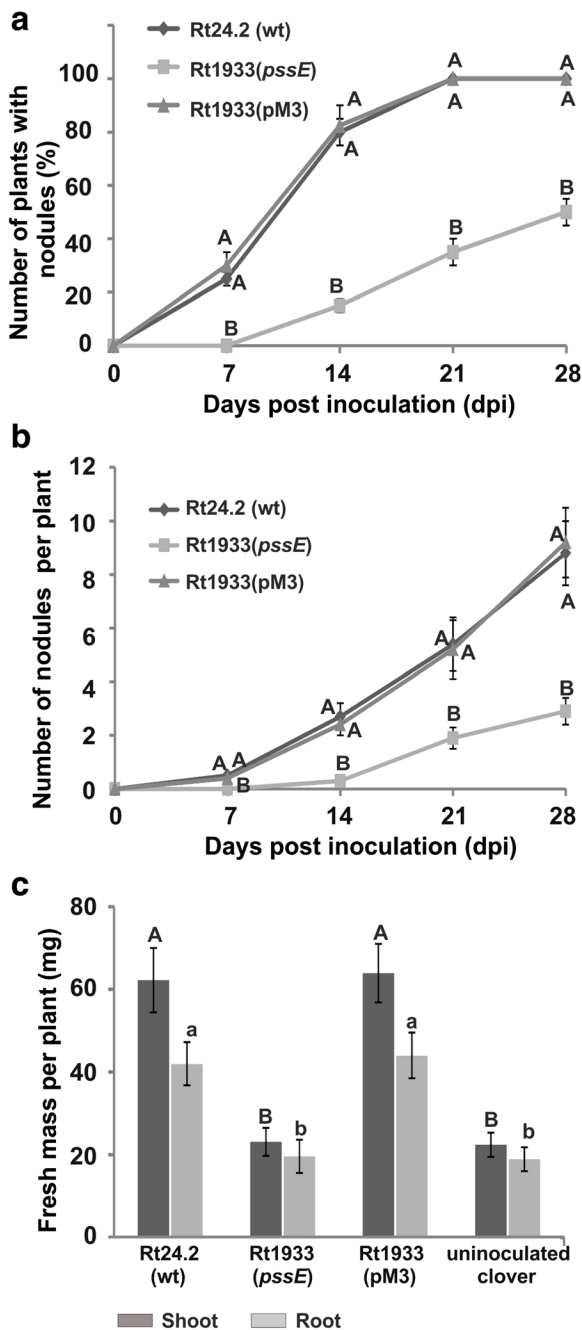
Fig. 4 Symbiotic properties of the wild-type *R. leguminosarum* bv. *trifolii* Rt24.2 and its Rt1933 and Rt1933(pM3) derivatives determined in a 4-week experiment using red clover (*Trifolium pratense*) as a host plant. **a** The percent of plants containing root nodules; **b** the average number of nodules elicited on clover roots by the tested strains; **c** the mass of shoots and roots of plants inoculated with the Rt24.2, Rt1933 and Rt1933(pM3) strains. The data are mean ± SD for three experiments. Differences statistically significant for tested strains are marked by different letters ($P < 0.05$; ANOVA, post hoc Tukey's test)

roots were performed using bacterial suspensions containing Rt1933 and live or temperature-killed Rt24.2 at a 1:1 ratio (Table 5). It was observed that when the clover roots were inoculated with a mixture containing live cells of both Rt1933 and Rt24.2 strains, the plants looked similar to those inoculated exclusively with Rt24.2 (i.e., they formed a large number of nodules effective in nitrogen fixation, which was confirmed by their high shoot mass). Furthermore, all the nodules were occupied by wild-type cells alone. These data confirm that the competitiveness of the *pssE* mutant was strongly decreased in comparison to that of the wild-type strain. In the case of co-inoculation of clover roots with the bacterial suspension containing live Rt1933 and dead Rt24.2 cells, significantly higher numbers of infected plants and nodules induced by Rt1933 were observed. These nodules, however, were white, irregularly shaped and ineffective in nitrogen fixation, as a consequence of which the plants were small, yellow and had a shoot mass similar to that of the plants inoculated with Rt1933 alone. Similar effects were observed for the plants inoculated with the bacterial suspension containing Rt1933 and EPS isolated from Rt24.2. The addition of purified EPS complemented the defect of the lack of EPS in the *pssE* mutant by improving the strain's effectiveness of nodule induction on host roots. This was confirmed by significantly increased numbers of plants forming nodules, nodules formed and mutant cells inside the nodules. However, also in this case, addition of the external EPS did not fully restore the wild-type symbiotic phenotype of the Rt1933 mutant. These data indicate that EPS derived from the wild-type strain complemented the defects of the EPS-deficient mutant *pssE* related to the only early stages of symbiotic interactions (i.e., induction of nodule formation and infection of host roots).

Discussion

Rhizobia comprise a diverse group of soil bacteria which establish nitrogen-fixing symbiosis with legumes (*Fabaceae*), a large and economically important group of plants that are a source of food, feed, and biofuels and are used in several other industrial applications (Olivares et al. 2013; Janczarek et al. 2015c; Gresshoff et al. 2015). Symbiotic interactions of this type are important for the functioning of the biosphere, since they provide large amounts of nitrogen to plants, increase soil fertility, and
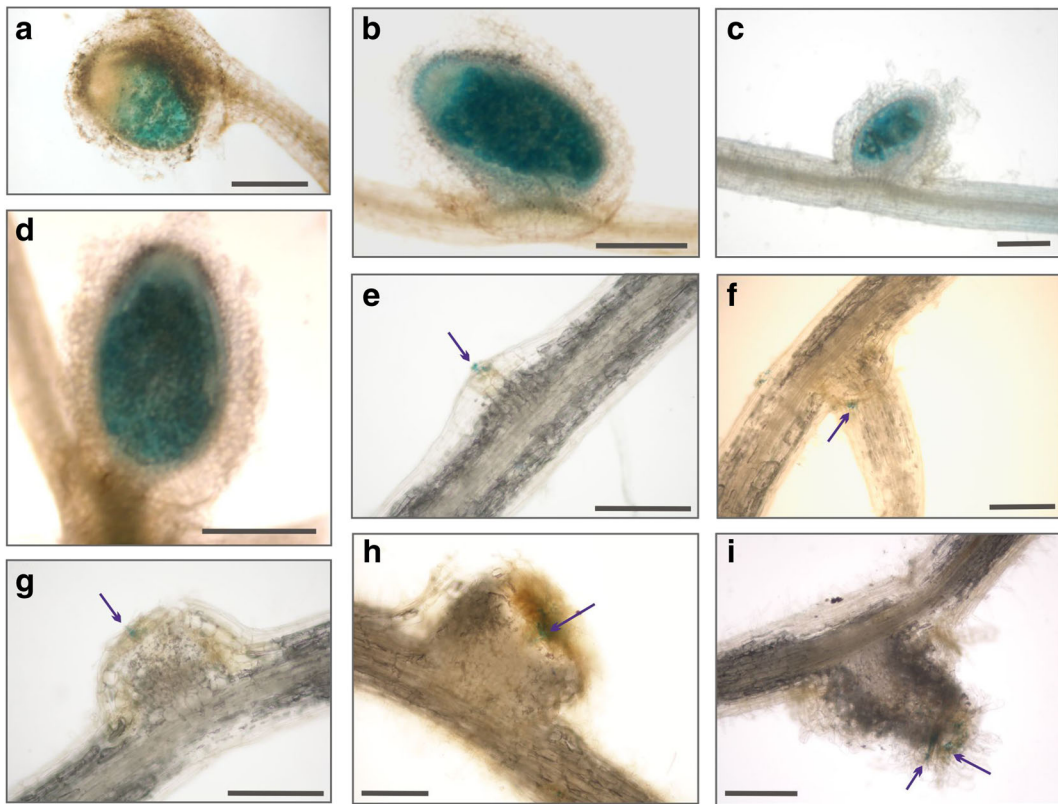
Fig. 5 Light microscopy of nodules elicited on clover roots (*Trifolium pratense*) by the wild-type *R. leguminosarum* bv. *trifolii* Rt24.2, the *pssE* mutant Rt1933, and the Rt1933(pM3) strains harboring the *gusA* reporter gene for β-glucuronidase. **a**, **b** Rt24.2 wild-type nodules at 7 and 21 days post inoculation (dpi), respectively; **c**, **d** 7-dpi and 21-dpi nodules containing Rt1933(pM3), respectively; (**e** - **i**) nodules induced by the Rt1933 mutant: **e** – a root after 7 dpi, **f** – a nodule primordium after 10 dpi, **g** – a 14-dpi nodule, **h** – a 21-dpi nodule, **i** – a 26-dpi nodule, respectively; bars = 0.2 mm (**a** and **c**), bars = 0.5 mm (**b** and **d**), bars = 0.3 mm (**e** - **i**). The nodules were stained for GUS activity

restore damaged ecosystems (Yanni et al. 1997; Kennedy et al. 1997; Qin et al. 2011; Angus et al. 2013). Recently, it has been established that rhizobial EPS plays a crucial role as a signal molecule in early stages of symbiosis (Kawaharada et al. 2015). Moreover, this polysaccharide is essential for adhesion, biofilm formation, and protection of bacterial cells against several stress conditions (Fujishige et al. 2006; Janczarek and Rachwał 2013). EPS synthesis in rhizobia is a multi-step process which involves the activity of several enzymes. In *R. leguminosarum*, a great majority of *pss* genes encoding proteins which participate in this biosynthetic pathway are located in the large chromosomal cluster Pss-I (Król et al. 2007; Sadykov et al. 1998; Janczarek et al. 2015b).

In this work, we demonstrated that the *exo99* mutation located in *pssE* of the Pss-I region affected EPS synthesis and cell-surface properties of *R. leguminosarum* bv.

trifolii, and the observed changes resulted in disturbances in the symbiotic interaction of this bacterium with clover plants (Fig. 1, Table 2).

It was established earlier that *pssD* and *pssE* genes were expressed from the same promoter and that large fragments of their ORFs overlapped (Sadykov et al. 1998; van Workum et al. 1997; Król et al. 2007; Janczarek et al. 2015b). Sadykov et al. (1998) have reported for *R. leguminosarum* bv. *viciae* strain VF39, which effectively infects *Vicia faba*, *V. sativa*, and *Pisum sativum,* that a mutation located at the 5′-end of *pssE* disturbs EPS synthesis. However, neither the precise site of this mutation nor the phenotypic properties of the mutant strain have been characterized. *pssD* mutations obtained earlier in strains of *R. leguminosarum* bv. *viciae* VF39, *R. leguminosarum* bv. *trifolii* RBL5599 and TA1 resulted in similar effects as the *pssE* mutation

Table 5 Effect of addition of purified EPS or Rt24.2 on nodulation of clover by Rt1933

Symbiotic parameter	Strains				
	Rt1933 (*pssE*)	Rt1933+ Rt24.2[a]	Rt1933+ Rt24.2 (dead)[a,b]	Rt1933+ EPS[c]	Rt24.2 (wt)
Plants forming nodules (%):					
week 1	0 ± 0[B]	28.0 ± 4.0[A]	0 ± 0 [B]	0 ± 0 [B]	29.8 ± 3.6 [A]
week 2	25.2 ± 3.0[C]	87.2 ± 6.3[A]	36.0 ± 4.3[B]	46.9 ± 3.9[B]	89.0 ± 4.8[A]
week 3	42.0 ± 4.1[C]	100 ± 0[A]	86.1 ± 4.0[B]	88.2 ± 4.5[B]	100 ± 0[A]
week 4	56.1 ± 4.9[B]	100 ± 0[A]	93.8 ± 4.9[A]	95.1 ± 4.7[A]	100 ± 0[A]
Number of nodules (plant^{-1})					
week 1	0 ± 0[B]	0.25 ± 0.05[A]	0 ± 0[B]	0 ± 0[B]	0.35 ± 0.1[A]
week 2	0.3 ± 0.2[B]	3.5 ± 0.8[A]	0.6 ± 0.2[B]	0.8 ± 0.3[B]	3.2 ± 0.7[A]
week 3	2.5 ± 0.7[C]	6.4 ± 1.0[A]	4.4 ± 0.8[B]	4.2 ± 0.9[B]	6.5 ± 0.9[A]
week 4	3.4 ± 0.9[B]	9.3 ± 1.5[A]	7.2 ± 1.2[A]	7.4 ± 1.3[A]	9.5 ± 1.6[A]
Fresh shoot mass (mg plant^{-1})[d]	24.8 ± 2.2[B]	56.3 ± 5.2[A]	25.5 ± 2.4[B]	25.9 ± 2.1[B]	59.1 ± 6.1[A]
Fresh root mass (mg plant^{-1})[d]	22.8 ± 2.3[B]	39.9 ± 3.1[A]	23.7 ± 2.4[B]	24.0 ± 2.6[B]	40.8 ± 3.3[A]
Nodule occupancy (×10^4)[d,e]	0 ± 0[C]	26.9 ± 5.4[A]	0.01 ± 0.003[B]	0.012 ± 0.004[B]	31.2 ± 6.2[A]

Means within the same rows for tested strain combinations followed by different capital letters have significantly different values (P 0.05)

[a] Bacterial suspensions contained Rt1933 and Rt24.2 strains at a 1:1 ratio [b] Suspension of Rt24.2 cells was heated for 10 min at 95 °C prior to use

[c] EPS isolated from Rt24.2 culture was added in the amount of 50 μg per plant

[d] The parameters were determined four weeks after plant inoculation. Values are means ± SD of the means for three experiments. Each experiment was conducted using 20 plants for each strain and treatment tested

[e] The values are means for 12 nodules

(i.e., a lack of EPS production and strong disturbances in nodule infection) (van Workum et al. 1997; Sadykov et al. 1998; Król et al. 1998).

The *R. leguminosarum pssD* and *pssE* genes encode proteins homologous to glycosyltransferases from the GTB-type superfamily. The amino acid sequences of PssD and PssE are similar to the N-terminal and C-terminal halves of the glucuronosyl-(β1,4)-glucosyltransferase SpsK of *Sphingomonas* S88, respectively (Sadykov et al. 1998; Ivashina and Ksenzenko 2012). This observation suggested that these proteins might represent two subunits of the same enzyme. As was proposed by Ivashina and Ksenzenko (2012), a catalytic domain was located in the PssE protein, whereas PssD was an integral inner membrane protein. They also found that integration of PssE into a bacterial membrane strongly depended on the presence of PssD and vice versa. In addition, Pollock et al. (1998) used complementation of the *Sphingomonas* S88 *spsK* mutant by *pssDE* of *R. leguminosarum* and an analysis of the sugars incorporated into lipid-linked disaccharides in permeabilized cells to demonstrate that these genes encoded proteins of glucuronosyl-(β1 → 4)-

glucosyltransferase activity. They also showed that glycosyltransferases which formed alternative sugar linkages to the same substrate inhibited polysaccharide synthesis or were deleterious or even lethal to the bacterium. This confirms the very high specificity of the enzymes participating in the synthesis of various EPS. Bacterial glycosyltransferases comprise a very large group of enzymes whose chief role in the biosynthesis of disaccharides, oligosaccharides, and polysaccharides is to catalyze the transfer of sugar moieties from activated donor molecules to other sugar molecules (Schmid et al. 2016).

We also showed in the present study that other properties of the *pssE* mutant were considerably changed in relation to the wild type (Fig. 3, Tables 3 and 4). This suggested that the lack of EPS in the mutant affected its cell-surface properties, whereas the slower growth of the mutant may have led to decreased motility, delayed root hair invasion, and consequently, a decreased nodule occupancy. Similarly to our findings, a higher sensitivity to SDS and DOC and oxidative stress factors (e.g., hydrogen peroxide) has been described for other rhizobial mutants defective in EPS synthesis, such as the *pssA*

and *pssD* mutants of *R. leguminosarum* and an *ORS571-X15* mutant of *Azorhizobium caulinodans* (Wielbo et al. 2004; D'Haeze et al. 2004; Janczarek and Rachwał 2013; Jaszek et al. 2014). These data indicate that the EPS surrounding bacterial cells plays an important protective role against several stress factors.

Moreover, we observed that the hydrophobicity of the *pssE* mutant was considerably changed in comparison with the wild type. This finding is in congruence with our previous data for an EPS-deficient *pssA* mutant, which showed significant differences in surface properties (i.e., electrokinetic mobility, surface charge, and hydrophobicity) compared to the wild-type strain (Janczarek et al. 2015a; Cieśla et al. 2016). Also, other researchers indicate that the presence of extracellular polysaccharides produced by various microorganisms has an important impact on their cell-surface properties (Lage et al. 2012; Planchon et al. 2013; Jttawuttipoka et al. 2013; Postma et al. 1991).

Furthermore, the *pssE* mutation affected the growth and motility of *R. leguminosarum* bv. *trifolii*. Decreased growth kinetics and swarming of Rt1933 in relation to Rt24.2 were observed in all of the tested growth media (Fig. 2, Table 3). This led us to the conclusion that slower migration of the *pssE* mutant in comparison to the wild type was probably caused by the decreased growth kinetics of the mutant cells. Tambalo et al. (2010) showed for two *R. leguminosarum* bv. *viciae* strains VF39SM and 3841 that parameters such as the type of growth medium, agar concentration, and temperature were essential for rhizobial motility. The EPS-deficient *pssA* mutant of *R. leguminosarum* bv. *trifolii* was also characterized by decreased motility in relation to the wild type. Similar findings have been reported in a study by Vanderlinde et al. (2009), in which a mutant strain of *R. leguminosarum* bv. *viciae* 3841 which did not contain the 27-hydroxyoctacosanoic acid modification in LPS was impaired in motility and biofilm formation. Moreover in *S. meliloti*, the presence of a regulatory network linking rhizobial EPS synthesis, motility and quorum sensing has been confirmed (Daniels et al. 2006; Sourjik et al. 2000; Glenn et al. 2007).

EPS is very important not only for free-living rhizobia, but also for the establishment of successful symbiosis with many legumes (e.g., clover, pea, vetch, and alfalfa) (Ivashina et al. 1994; Cheng and Walker 1998; Janczarek and Rachwał 2013). As reported recently for *M. loti–L. japonicus* symbiosis, this polysaccharide plays a crucial role as a signal which is recognized and bound by a plant receptor (Kawaharada et al. 2015; Kelly et al. 2013; Muszyński et al. 2016).

Finally, we found that the lack of EPS and changes in cell-surface properties caused by the *pssE* mutation significantly affected the symbiosis of *R. leguminosarum* bv. *trifolii* with red clover (Figs. 4 and 5). The effectiveness of induction of root nodules by the Rt1933 mutant was strongly decreased. The non-numerous nodules appeared on the roots with a large delay, and even four weeks into the experiment, only ~50% of the plants had nodules. Moreover, these nodules were not occupied by *pssE* mutant cells, and, in consequence, did not fix nitrogen. The symbiotic phenotype of Rt1933 confirms that the PssE protein plays an essential role in EPS synthesis and symbiotic interactions.

In our further experiments, we studied the influence of EPS on symbiotic interactions of the *pssE* mutant with red clover (Table 5). The results we obtained showed that the addition of wild-type EPS, either in the form of purified EPS isolated from the Rt24.2 culture or by co-inoculation with temperature-killed Rt24.2 cells, significantly affected the early stages of symbiosis, as manifested by the higher numbers of plants forming nodules and higher number of nodules induced by the EPS-supplemented Rt1933 in comparison to the plants inoculated with Rt1933 alone. However, the addition of EPS did not restore the full symbiotic phenotype of this mutant (i.e., its ability to fix nitrogen). In contrast, Djordjevic et al. (1987) showed for some EPS-deficient mutants of *S. fredii* NGR234 that the addition of a homologous EPS restored not only the infection effectiveness but also the nitrogen fixation ability of these strains in symbiosis with *Leucaena* and *Macroptilium atropurpureum* (25% to 40% of the plants formed Fix[+] nodules, depending on the type of mutant studied). Those authors also observed partial restoration of the symbiotic properties of the EPS-deficient mutant of *R. leguminosarum* bv. *trifolii* ANU843 when wild-type EPS was added (25% of the tested white clover plants formed Fix[+] nodules). These data suggest that the ability of EPS to complement symbiotic defects of EPS-deficient mutants may depend on various factors, such as the type of mutation, the kind of bacterium, and the kind of host plant. On that basis, we hypothesized that in the case of the *pssE* mutant, apart from the absence of EPS, its effectiveness in host root infection and nodule occupation was additionally negatively affected by the slower growth and cell motility of this strain.

EPS plays an essential role in the attachment of rhizobia to plant roots, the formation of biofilm on the root surface and host plant infection (Jones and Walker 2008; Williams et al. 2008; Rinaudi and Giordano 2010). This polysaccharide forms the most external layer of cells with little or no associations with the bacterial envelope, in contrast to capsular polysaccharide, which usually has a very similar structure to EPS, but is associated with the bacterial outer membrane (Downie 2010). Both of these polysaccharides (and especially EPS) are required for the initiation and propagation of infection threads – special structures via which rhizobial cells invade host roots and reach nodule tissues. EPS is an essential component of the infection thread matrix and forms a protective layer which prevents penetration of toxic H_2O_2 generated by host plants during their invasion (D'Haeze et al. 2004; Jones and Walker 2008). D'Haeze et al. (2004) showed that an ORS571-X15 mutant of *Azorhizobium caulinodans* which produced considerably less EPS than the wild type, was more sensitive to oxidative stress and induced ineffective nodules on its host plant, *Sesbania rostrata*.

It has traditionally been accepted that rhizobial EPS plays a much more important role in symbiotic relationships in which macrosymbionts form indeterminate nodules, such as *Medicago–S. meliloti*, *R. leguminosarum* bv. *trifolii–Trifolium* and *R. leguminosarum* bv. *viciae–Vicia/Pisum* (Cheng and Walker 1998; Rolfe et al. 1996; Ivashina et al. 1994, van Workum et al. 1997). In these cases, EPS-deficient mutants of these rhizobial species have proved to be defective in nodule invasion, and the nodules formed were unable to fix nitrogen. However, this is not always the case, since an EPS-deficient *exoA* mutant of *S. fredii* HH103 has been shown to be able to induce nitrogen-fixing nodules on two plants which formed indeterminate nodules (*G. uralensis* and pigeon pea) (López-Baena et al. 2016).

It had been reported previously that mutations in *pssA*, *pssD*, and *pssS* genes of *R. leguminosarum* bvs. *trifolii* and *viciae* totally abolished EPS synthesis and led to strong disturbances in symbiosis with compatible host plants (sporadically formed nodules were small and hardly occupied by bacterial cells) (Ivashina et al. 1994; Rolfe et al. 1996; van Workum et al. 1997; Sadykov et al. 1998; Ivashina and Ksenzenko 2012; Janczarek et al. 2015b). Similarly, EPS-deficient mutants of *S. meliloti* harboring mutations in genes which encode glycosyltransferases (*exoY*, *exoA*, *exoL*, *exoM*, *exoO* and *exoW*) induced non-nitrogen-fixing nodules on alfalfa

(Cheng and Walker 1998; Reuber et al. 1991; Becker et al. 1993a, b). However, not only the absence of EPS but also alterations in its structure might lead to severe impairment in symbiosis. For example, a lack of pyruvyl residues in subunits caused by a *pssV* mutation abolished succinoglycan production in *S. meliloti* and negatively affected its symbiosis with the host plant (Glucksmann et al. 1993; Becker et al. 1993b). Mutations in *exoC* and *exoB* genes involved in the synthesis of the sugar precursors glucose-1-phosphate and UDP-galactose, respectively, abolished the production of succinoglycan and other polysaccharides containing galactose (galactoglucan and LPS) (Glucksmann et al. 1993; Becker et al. 1993a, b). Furthermore, recent data have shown that succinyl groups in succinoglycan are critical for successful host root invasion by *S. meliloti*, whereas the chain length of this polymer has much less effect on its role in symbiosis (Mendis et al. 2016). An *exoH* mutant, which produced large amounts of succinoglycan but lacked succinyl residues, was unable to invade alfalfa roots.

In conclusion, all these data show that rhizobial EPS is essential for successful invasion of legume roots and effective nitrogen fixation. Mutations in the genes encoding glycosyltransferases which participate in EPS unit assembly and proteins responsible for sugar precursor synthesis cause similar defects, i.e. a disruption in EPS synthesis and disturbances in the establishment of symbiotic interactions.

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