Introduction

Polysaccharides are abundant components of bacterial cells as well as the matrices that they form in their ecological niches. Exopolysaccharides (EPS) are extracellular polysaccharides secreted by many bacteria that play several physiological roles. EPS produced by rhizobia protect bacteria from adverse conditions in the demanding environment of soil and are among the most important factors determining a successful symbiotic interaction between rhizobia and leguminous plants [1,2]. Rhizobia living in the rhizosphere attach to the plant roots, invade plant tissues and colonize cells of the forming nodule, where they differentiate into bacteroids which provide fixed nitrogen for the plant in exchange for carbon [3]. Polysaccharide synthesis, independent of the glycoform produced, is a multistep process that employs several enzymatic and structural proteins. Generally, polysaccharides may be completely assembled in the cytoplasm before being targeted to the periplasm, where they are subsequently polymerized in the periplasm and transported to the external environment. Type 1 and 4 capsular polysaccharides of Escherichia coli, EPS and most O-antigens of Gram-negative bacteria follow the second pathway, i.e. assembly of the repeating unit in the cytoplasm and polymerization thereof in the periplasm combined with translocation outside the cell. This pathway is called the Wzx/Wzy-dependent pathway, as it requires the Wzx flippase and Wzy polymerase, unlike systems involving ABC-transporters or synthase proteins [4,5].

Polysaccharide biosynthesis is initiated by glycosyltransferases involved in the assembly of the repeating unit on the lipid carrier undecaprenyl pyrophosphate. Complete subunits are then translocated to the periplasmic face of the inner membrane by the Wzx flippase [6,7] and then polymerized by the Wzy polymerase to the extent that is regulated by the Wzz co-polymerase (Wzz in the case of EPS and O-antigens) [8]. The nascent chain is translocated outside the cell by the Wza oligomeric channel protein [9–11]. Several genetic and structural studies revealed complex interrelations between proteins engaged in these processes, e.g. Wzx-Wzy/Wzz [12], Wzy-Wzz [13,14], Wzc-Wza [15], as well as between glycosyltransferases [16,17].

Proteins engaged in EPS synthesis in R. leguminosarum bv. trifolii are encoded within the chromosomal Pss-I region. The region comprises genes encoding glycosyltransferases [18,19], a
putative flippase [20], a polymerase [21], a co-polymerase [22], and an outer membrane channel protein [23,24]. The functions of several (but not all) glycosyltransferases encoded within the region were previously dissected. Glucosyl-IP-transferase PssA is the priming glycosyltransferase initiating the assembly of the octasaccharide EPS unit (Figure 1) by the transfer of UDP-glucose to the undecaprenyl phosphate lipid carrier attached to the cytoplasmic membrane [19,25]. In the subsequent step, a glucuronosyl-(β-1,4)-glucosyl transferase composed of PssD and PssE catalyses the addition of a glucuronic acid residue [18,26]. The addition of the second glucuronic acid is mediated by the glucuronosyl-β-1,4-glucuronosyltransferase PssC [18,25,26]. The outcome of mutations in pssA, pssD, pssE, and pssC is pleiotropic and in addition to abolishing the capacity to synthesize EPS, it affects the level of synthesis of several cellular proteins [27]. PssL is homologous to Wzx and was proposed to function as a flippase that translocates EPS subunits to the outer leaflet of the inner membrane [20]. PssT is homologous to Wzy and serves as a polymerase of EPS subunits; the pssT mutant produced EPS with a greater amount of high-molecular-weight (HMW) EPS than the wild type [21]. Polymerization of polysaccharides is influenced by a protein assigned to a family of polysaccharide co-polymerases (PCP) [28] that are distinguished by their common membrane topology with a large periplasmic loop flanked by two transmembrane segments [29]. PssP was demonstrated to be a PCP protein. It is a large inner membrane protein comprising a periplasmic domain with coiled-coils and two transmembrane segments [22,30]. It was shown to be indispensable for EPS synthesis [22]. The terminal stage in the assembly of EPS, i.e. the translocation of a polymer across the outer membrane, occurs through the pore formed by the PssN lipoprotein homologous to Wza protein [23]. Bacterial two-hybrid (BTH) assays provided evidence for interactions between proteins involved in EPS biosynthesis and transport, namely PssP-PssT and PssP-PssN, consistent with the notion of a multicomponent complex [30].

Pss-I is likely not the only gene cluster involved in polysaccharide synthesis in R. leguminosarum RtTA1; several other regions with candidate genes were identified both in the chromosome and on a plasmid [31]. One of them, the chromosomal Pss-II region (GenBank Accession No. DQ384109), is comprised of several genes encoding putative homologues to constituents of the Wzx/Wzy pathway, suggesting involvement in the synthesis of EPS or other polysaccharide(s). One of the proteins encoded within the Pss-II cluster is PssP2. Its primary and predicted secondary structure similarity, protein topology, and subcellular localization resembled PCP proteins and indicated a possibility of PssP2 engagement in the synthesis of LPS and/or EPS.

To examine PssP2 involvement in the synthesis of either EPS or LPS, a mutant disrupted in the pssP2 gene was constructed. The significance of this qualitative change in the PssP2 protein for production of EPS and LPS and the symbiotic phenotype was studied. Moreover, the interrelations between PssP2 and thus far characterized Pss proteins were also examined. The results obtained indicate that the PssP2 protein is yet another component of the protein complex that plays an important role in EPS chain-length determination.

Materials and Methods

Bacterial strains and culture conditions

Strains used in this work are listed in Table 1. Escherichia coli strains of general use were grown in lysogeny broth (LB) medium at 37°C [32], and Rhizobium strains were grown in TY [32], M1 with 1% glycerol [32] or 79CA with 1% mannitol or 1% glycerol at 28°C [33]. Bacterial two-hybrid (BTH) complementation assays were performed with the E. coli cya strain DH112, which was grown at 30°C. Antibiotics were used at following final concentrations: ampicillin 100 μg/ml, kanamycin 40 μg/ml, gentamycin 5 (E. coli) or 10 μg/ml (Rhizobium), tetracycline 10 μg/ml and rifampin 40 μg/ml.

Plasmid constructions for mutagenesis, promoter probing and pssP2 overexpression

Plasmids and primers used in this work are listed in Table 1. Standard protocols for genomic DNA isolation, PCR, molecular cloning, transformation and DNA analysis were used [32]. pKP2 plasmid used for integration mutagenesis of pssP2 gene was constructed by subcloning of the PstI-SalI fragment of pssP2 gene into the pK19mobGII vector [34]. Promoter probe constructs were produced from cloning of PCR products covering putative pssP2 and pssP promoters into the BluntII-PstI restriction sites of pMP220 vector [35]. pQBP2his plasmid encoding a His-tagged version of PssP2 was constructed using pQE30 expression vector (Qiagen) and pBR1MCS-5 [36]. For that purpose, pssP2 was amplified with Pfu polymerase (Thermo Scientific) using P2exFWSacI and P2exRVHindIII primers. The amplicon was cloned between the SacI and HindIII restriction sites in pMP220 vector. The resulting construct was used for chromosomal integration into the C genome of Rhizobium

Construction of RtTA1 chromosomal insertion mutant pssP2:pKP2

pKP2 plasmid was transferred from E. coli S17-1 to RtTA1 by conjugation and transconjugants were selected on 79CA medium with kanamycin. Bacterial mating experiments were performed as described by Simon et al. [37]. The clones with pK19mobGII chromosomal integration were selected on 79CA medium supplemented with 5-bromo-4-chloro-3-indolyl-β-D-glucuronide substrate for β-glucuronidase (GUS). The selected kanamycin-resistant and GUS-negative clones were probed for genominc rearrangements by PCR with primers matching the pssP2 gene and pUC universal primers matching vector sequences.
### Table 1. Strains, plasmids and oligonucleotide primers used in this work.

| Strain, plasmid or primer | Relevant description | Source or reference |
|---------------------------|----------------------|---------------------|
| **Escherichia coli**      |                      |                     |
| JM101                     | Δlac proAB thi supE F’ traD36 proAB lacZ51 ΔM15S        | [32]               |
| S17-1                     | 294 derivative RP4-2-Tc::Mu-Km::Tn7 chromosomally integrated | [37]               |
| DH5α                      | supE44 ΔlacU169 (Δ80 lacZΔM15S) hisD17 recA1 endA1 gyrA96 thi-1 relA1 | [32]               |
| DHM1                      | Reporter strain for BTH system; F R 1 glnV44(AS) recA1 endA1 gyrA96 thi-1 hisD17 spo71 rfbD1 cya-854 | [62]               |
| **Rhizobium leguminosarum bv. trifolii** |                      |                     |
| 78-2                      |                      |                     |
| **Plasmids**              |                      |                     |
| pK19mobGII                | pUC19 derivative, lacZ, mob, gusA; Km’                  | [34]               |
| pKP2                      | pK19mobGII with 482-bp PstI-SalI fragment of pssP2 gene | This work           |
| pMP220                    | lacP, mob, promoterless lacZ, Tc’                         | [35]               |
| pMP01                     | pMP220 with 681-bp EcoRI fragment carrying pssO promoter | [38]               |
| pMP2P                     | pMP220 with 1.1-kb BamHI-PstI fragment of pARF136 carrying the pssP promoter | [22]               |
| pMP-P2                    | pMP220 with 245-bp BglII-Xhol fragment carrying putative pssP2 promoter | This work           |
| pMP-Y                     | pMP220 with 424-bp BglII-Xhol fragment carrying putative pssY promoter | This work           |
| pUT18                     | Two-hybrid plasmid for cyaAT8 fusion construction, Amp’       | [62]               |
| pUT18C                    | Two-hybrid plasmid for cyaAT8 fusion construction, Amp’       | [62]               |
| pKT25                     | Two-hybrid plasmid for cyaAT25 fusion construction, Km’         | [62]               |
| pKT25C                    | Two-hybrid control plasmid                                    | [62]               |
| pK19mobGII pUT18          | Two-hybrid plasmid containing cyaAT8-pssP, pssT, pssL fusion, respectively | [30]               |
| pK19mobGII pUT18C         | Two-hybrid plasmid containing cyaAT8-pssP, pssT, pssL fusion, respectively | [30]               |
| pK19mobGII pKT25pssP2    | Two-hybrid plasmid containing cyaAT25- pssP2, pssA, pssC fusion, respectively | This work           |
| pK19mobGII pK19mobGII pssP2/psst/pssL | Two-hybrid plasmid containing cyaAT8-pssP, pssT, pssL fusion, respectively | [30]               |
| pK19mobGII pK19mobGII pssP2/pssA/pssC | Two-hybrid plasmid containing cyaAT25- pssP2, pssA, pssC fusion, respectively | This work           |
| pQ9040                    | Expression vector, Amp’                                      | Qiagen              |
| pBRI1MCS-5                | mob Gm’                                            | [36]               |
| pQ9040/22his              | pQ9040 with pss2 gene devoid of its own ATG and STOP codons cloned into SacI-HindIII | This work           |
| pQBP2his                  | pBRI1MCS-5 with expression cassette from pQ9040/22his cloned into Xhol-HindIII | This work           |
| **Primers**               |                      |                     |
| CFwBTH                    | 5’ AAATCTAGAATACGAAAAAGCCTTTTCGCCAT3’                    | amplification of the pssC gene for the BTH system (XbaI recognition site) |
| CRvBTH                    | 5’ AAAGCAGCTTGGGCGCATTGGTTGTTATGC3’                       | amplification of the pssC gene for the BTH system (BamHI recognition site) |
| AFwBTH                    | 5’ AAAGTCTAGAAGGTAAACCATTGATCGGCTATTGC3’                  | amplification of the pssA gene for the BTH system (Sall recognition site) |
| ARvBTH                    | 5’ AAAGATCCAGGCTTTACCAGCGTGCACTCCGAC3’                     | amplification of the pssA gene for the BTH system (XbaI recognition site) |
| AFwBTH2                   | 5’ AAATCTAGAGACGGTTAACCATTGATCGGCTA3’                      | amplification of the pssA gene for the BTH system (XbaI recognition site) |
| P2FwBTH                   | 5’ AAATCTAGAAGCCTAAAGCAGATCCTACCGGCT3’                    | amplification of the pssP2 gene for the BTH system (XbaI recognition site) |
Promoter activity assays

pMP-P2 and pMP-Y plasmids carrying putative promoters of \( pssP2 \) and \( pssY \) genes were introduced into \( R\). \( T\). \( T\). \( A\)1 by electroporation. Activities of promoters were measured in cultures of respective strains grown in TY, 79CA with 1% mannitol or M1 supplemented with tetracycline resistance; Amp \( r \), ampicillin resistance; Gm \( r \), gentamicin resistance.

Co-purification of interacting proteins

The level of His\(_6\)-PssP2 production in \( E\). \( c\). \( o\) JM101 carrying pQP2his was very low and the protein was difficult to identify through Western blotting with anti-His antibodies. pQP2his plasmid was introduced to the \( R\). \( p\)2-1.1 integration mutant by electroporation. Both the mutant and its complemented derivative were subjected to in vivo cross-linking with 0.5% formaldehyde according to the procedures described previously [23,30]. Afterwards the cells were washed and resuspended in 50 mM sodium phosphate, 300 mM sodium chloride buffer, pH 7.0 and disrupted in a French press (one passage at 18,000 psi). Crude lysate was clarified to remove unbroken cells and debris by centrifugation at \( 6000 \times g \) at 4°C and the supernatant was ultracentrifuged at \( 85,000 \times g \) for 1 h to sediment the membranes. The membranes were resuspended in 50 mM sodium phosphate, 300 mM sodium chloride buffer, pH 7.0 and supplemented with DDM (n-dodecyl-beta-D-maltoside; Sigma) to a final concentration of 0.2%. Membrane proteins were solubilized in a cold room for 1 h. Then, the mixture was centrifuged at \( 14,000 \times g \) for 30 min, the supernatant was collected and mixed with TALON metal affinity eluted using 50 mM sodium phosphate, 300 mM NaCl, 150 mM imidazole buffer, pH 7.0. Samples were collected and analyzed by SDS-PAGE and Western immunoblotting with anti-His and anti-PssP antibodies.

Plant tests

Red clover (\( T\). \( p\)ratense \( L\). cv. Rozeta) seeds were surface sterilized, germinated, and grown as described previously [39]. Four-day-old clover seedlings were planted in sterile nitrogen-free
slants (one per tube) and allowed to grow for 4 days before inoculation with 0.2 ml of cell suspension at an approximate density of 1.0×10^9 cells/ml. Five weeks after the inoculation, plants were harvested and examined for nodulation and nitrogen fixation phenotype. Nitrogen fixation was evaluated indirectly, on the basis of color and green matter production, which was estimated by weighing the shoots. Twenty clover plants were used for each strain.

**Cell-surface polysaccharide analysis**

Extracellular exopolysaccharides were precipitated with 5 volumes of ethanol from the supernatants of bacterial cultures grown with shaking for 3 days in 100 ml of 79CA medium with 1% glycerol. EPS was fractionated twice on 0.7 cm × 90 cm Sepharose CL-6B column (Sigma-Aldrich). Fractions of 1 ml were collected and the total sugar content was determined according to Yasar [40] and calculated in glucose equivalents. Obtained results were averaged. Molecular weight markers used were: Blue Dextran, 2 MDa; Dextran T550, 550 kDa, and Dextran T10, 10 kDa. The glycosyl composition analysis of EPSs were determined by preparation of the alditol acetate derivatives, identified and quantified by gas liquid chromatography mass spectrometry (GLC-MS). The samples were hydrolyzed (120°C, 2 h) in 2 M trifluoroacetic acid (TFA) reduced with sodium borodeuteride (NaBD₄), and acetylated. To confirm the presence of uronic acids, methanolysis and carboxyl reduction with NaBD₄ prior to TFA hydrolysis was performed [41]. LPS from *Rhizobium leguminosarum* strains was isolated by whole cell microextraction using proteinase K digestion as described by Apicella [42]. Electrophoresis was carried out on a 12.5% SDS-PAGE polyacrylamide gel using a tricine buffer system [43] and visualised by oxidative silver staining according to the method of Tsai and Frasch [44].

**Sedimentation/autoaggregation analysis**

For sedimentation analyses the method described by Sorroche et al. [45], with minor modifications, was employed. *Rhizobium* strains were grown in 79CA medium with 1% mannitol at 28°C for 24 h, diluted to the same OD₆₀₀ 0.1 and grown for 48 h. Afterwards, 5 ml of the cultures were transferred to agglutination slants (one per tube) and allowed to grow for 4 days before incubation without agitation for 24 h at 4°C. Next, OD₆₀₀ was measured for 0.5 ml of the upper layer of suspension (Ax). The autoaggregation percentage was expressed as follows: 1−(Aₐ/Aₓ)×100 [45], averaged from five independent experiments and subjected to statistical analyses.

**Analyses of proteins**

Proteins were analyzed by SDS-PAGE and either visualized by PageBlue Staining Solution (Thermo Scientific) or electroblotted onto PVDF membrane (Millipore). Immunoblots were probed with the primary: anti-PssP [30] and anti-His antibodies (Roche) and secondary anti-rabbit and anti-mouse IgG antibodies conjugated with alkaline phosphatase (Sigma).

**BTH testing**

The bait/’prey’ vectors pKT25, pUT18C and pUT18, and the control plasmids pKT25-zip and pUT18C-zip were used in protein interaction analyses. To construct plasmids encoding Pss-CyaA fusion proteins, pssP2, pssA and pssC genes were PCR amplified using appropriate primers listed in Table 1, with RiTAI genomic DNA as the template. Amplified DNA fragments were digested with appropriate restriction enzymes (Thermo Scientific), the names of which are indicated alongside the names of primers used for amplification in Table 1, subcloned into the corresponding sites of the pKT25, pUT18C and pUT18 vectors and transformed into *E. coli* DH5α strain. Constructs were verified by sequencing. Plasmids were then transformed into *E. coli* DH1 reporter strain, with selection on LB agar medium containing ampicillin, kanamycin, X-gal and IPTG. For a quantitative measurement of interaction strength β-galactosidase activity was measured as described previously [30]. The assay was performed in single tubes format. Construction of BTH plasmids with pssT, pssP and pssL genes was described previously [30].

**Bioinformatic analyses**

Putative homologues to PssP2 were identified via BLASTp [46]. HHpred tool was used to search against existing structures of PCP proteins in the PDB (Protein Data Bank) [47]. For the alignment of PssP and PssP2 ChsStaW [48] was used and the result was visualized with Alignment Viewer. HHpred and alignment tools are available at http://toolkit.tuebingen.mpg.de. Searching for tyrosine kinase motifs was assisted by BYKdb [49]. Protein subcellular localization was predicted with PSORTb [50]. Protein topology was analysed using: DAS [51], TMHMM [52], HMMTOP [53] and Phobius [54]. Coiled-coil regions were predicted with COILS [55] and NetPred 2.0 server [56] was used to predict phosphorylation sites. Putative promoters were predicted with Neural Network Promoter Prediction [57] and Rhizobium independent terminators were searched for using ARNold tool [58].

**Statistical analyses**

The results of promoter activity assays, plant tests, autoaggregation assays as well as bacterial two-hybrid results were submitted for statistical analyses, which were performed with STATISTICA software, using one-way analysis of variance (ANOVA) and the Tukey test at a significance level of p<0.05.

**Results**

PssP2 is a homologue of polysaccharide co-polymerases

The chromosomal Pss-II region in *Rhizobium leguminosarum* bv. *trifolii* TA1 (RiTAI) [31] is located ~200 kb from Pss-I and comprises several genes encoding putative proteins similar to proteins engaged in the model Wzx/Wzy-dependent pathway of polysaccharide assembly, suggesting involvement in the synthesis of EPS or other polysaccharide(s) in this strain (Figure 2A). One of the genes in the region, pssP₂, encodes a hypothetical protein (586 amino acids) similar to the PssP co-polymerase encoded within the Pss-I region. The local level of identity/similarity between PssP2 and PssP (scored with BLASTp) was shown to be 28%/47% in the region covering 256–554 aa of PssP2 and 27%/45% in the region covering 16–251 aa of PssP2 (Table 2). BLASTp searches throughout the UniProtKB/SwissProt database revealed similarity of PssP2 to known bacterial kinases involved in polysaccharide production (Table 2). BLASTp searches among non-redundant protein sequences revealed hits with prevalence of putative sugar transporter proteins as well as LPS and EPS biosynthesis proteins in *Rhizobium* (thus showing that the genes homologous to pssP2 are common in this group of bacteria). The most similar sequences were found in *R. leguminosarum* and *R. etli* (the lowest identity/similarity ~80%/90%), while the least similar sequences originated from the *R. phaseoli* species and genera *Mesorhizobium*, *Sinorhizobium* (*Ensifer*), and *Bradyrhizobium* (up to ~30%/40% identity/similarity; all the sequences were similar in length).
Figure 2. Organization of genes in the Pss-II region, constructs used in the pssP2 gene functional analyses and the results of PssP2 protein amino acid sequence analyses. A) Physical and genetic map of the R. leguminosarum bv. trifolii Pss-II region; genes encoding putative proteins similar to elements of the Wzx/Wzy-dependent polysaccharide polymerization pathway are indicated above the map; Rho-independent terminator predicted downstream orf5 gene is marked with a black rectangle, promoters predicted between the pssY and pssP2 genes are marked with red rectangles; B) Constructs used for integration mutagenesis of the pssP2 gene (pKP2; green bar) and probing putative promoters identified upstream the pssP2 (pMP-P2) and pssY (pMP-Y) genes (blue bars). Small black rectangles mark positions of primers used for amplification of promoter regions. Red rectangles below the pMP-P2 and pMP-Y constructs mark positions of identified promoters and the scores obtained for each predicted promoter. C) Genomic organization of the integration mutant pssP2::pKP2. Position of the plac promoter (red rectangle) in the vector part and the direction of transcription from the promoter are shown. D) Sequence alignment of PssP2 and PssP proteins of R. leguminosarum bv. trifolii TA1. The alignment was produced in ClustalW and visualised by Alignment Viewer; amino acids were coloured according to their biochemical properties, thus the same colour means either identity or similarity, e.g. positively charged amino acids Arg and Lys are marked in red. E) Scheme of PssP2 topology and specific motifs found in silico. Blocks representing domains are aligned respective to the location in the polypeptide; TMS, transmembrane segment.

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The hypothetical PssP2 amino acid sequence is characterized by the presence of several motifs: the Wzz motif (Pfam: PF02706) between 25–210 aa, found in chain length determinant proteins involved in lipopolysaccharide biosynthesis [PCP-1 family of proteins] as well as in bacterial tyrosine kinases (PCP-2 family of proteins), the G-rich domain (PF13007) on putative tyrosine kinase (between 257–337 aa), and the AAA-domain (ATPases Associated with diverse cellular Activities) (PF13614) between 409–537 aa (Figure 2E). Even though proteins similar to PssP2 and with an experimentally verified function are bacterial kinases, PssP2 probably does not have a kinase activity. BYKdb-assisted analysis experimentally verified function are bacterial kinases, PssP2 (domain of PssP2) (sequences clearly shows that there is a huge gap in the periplasmic group of proteins, i.e. the Walker A, A', and B as well as the C-terminal Y-cluster, found in the model Wzx protein [59]. On the contrary, the PssP protein (Pss-I region) possesses all motifs specific for this class of proteins except for the C-terminal Y (tyrosine)-cluster [22]. NetPhos analysis revealed that PssP2 might be phosphorylated on serine (25 sites), threonine (7 sites), or tyrosine (2 sites).

PssP2 is much smaller than PssP (their lengths are 586 aa and 746 aa, respectively) (Table 2) but its topology resembles Wzx-like proteins, not Wzx-like proteins. The PssP2 periplasmic domain is much shorter than in PssP (alignment of the two sequences clearly showed that there is a huge gap in the periplasmic domain of PssP2) (Figure 2D), and contrary to Wzx proteins, PssP2 possesses a long cytoplasmic domain yet devoid of specific kinase motifs.

Similarly to its homologues, PssP2 was predicted to be an inner membrane-embedded protein (PSORTb) with two transmembrane domains, the corresponding region of PssP2 was predicted with lower confidence) (COILS) (Figure 2E) but its topology resembles Wzx-like proteins, not Wzx-like proteins. The PssP2 periplasmic domain is much shorter than in PssP (alignment of the two sequences clearly showed that there is a huge gap in the periplasmic domain of PssP2) (Figure 2D), and contrary to Wzx proteins, PssP2 possesses a long cytoplasmic domain yet devoid of specific kinase motifs.

Multiple sequence alignment of PssP2 and homologous proteins listed in Table 2 (Figure S1) as well as search against protein structures deposited in PDB with HHpred tool (Table 3) revealed that the C-terminal domain of PssP2 may have a conserved fold resembling that of bacterial protein kinases. Moreover, despite the small number of published X-ray crystal structures of PCP periplasmic domains, the corresponding region of PssP2 was matched against PDB structures 4E29, 3BIO and 3B8M (Table 3). Together, these results provide several lines of evidence supporting homology of PssP2 to PCP proteins.

### Table 2. Putative homologues of PssP2 protein (586 aa) (ABD36550) identified through BLASTp searches.

| Homologous protein (aa) | Bacterium | Identity/similarity (%) (query coverage) | Accession number | Function | Reference |
|--------------------------|-----------|----------------------------------------|------------------|----------|-----------|
| ExoP (786)               | Sinorhizobium meliloti | 29/44 (207–578) 26/42 (38–251) | P33698 | Succinoglycan biosynthesis transport protein ExoP | [86] |
| Wzc (720)                | Escherichia coli | 22/41 (232–583) | P76387 | Tyrosine-protein kinase Wzc | [73] |
| CpsD (232)               | Streptococcus agalactiae | 29/43 (406–584) | Q3K0T0 | Tyrosine-protein kinase CpsD | [87] |
| Ptk (733)                | Acinetobacter johnsonii | 23/39 (219–578) | O52788 | Tyrosine-protein kinase Ptk | [88] |
| Etk (726)                | Escherichia coli | 21/35 (255–583) 26/43 (181–310) | P58764 | Tyrosine-protein kinase Etk | [89] |
| PssP (746)               | Rhizobium leguminosarum bv. trifoli T1A | 28/47 (258–554) 27/45 (16–236) | ABD47316 | Protein involved in EPS chain length determination | [22,24] |

The database used above was the non-redundant UniProtKB/SwissProt. The multiple alignment of the above mentioned sequences is presented in Figure S1 (Supplementary data).

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Transcriptional activity of the pssP2 gene

Organizations of genes in the Pss-II region, i.e. their orientation and spacing between putative ORFs, suggested that they formed two transcriptional units with promoters located between pssY encoding a putative glycosyltransferase and crp1 and active in both directions (Figure 2A). Analysis of the 5’-upstream region of pssP2 revealed that the gene might be preceded by a weak promoter (predicted with 0.85 score by Neural Network Promoter Prediction) localized in the 3’-end of the preceding pssY gene (Figure 2B). Stronger promoters were predicted to be localized upstream of the pssY gene (two promoters with the highest scores, 0.97 and 0.99, are marked in Figure 2B).

To determine whether the predicted pssP2 weak promoter is active in RtTA1, a DNA fragment covering this hypothetical promoter was PCR-amplified and cloned into pMP220 resulting in pMP-P2 (Figure 2B, Table 1). The level of β-galactosidase activity measured in RtTA1 was not different from that of the pMP220 vector alone (Figure 3). Taking into account lack of detectable activity, an additional fragment preceding the pssY gene was cloned into pMP220 to give pMP-Y (Figure 2B, Table 1) to assess whether there is a promoter that might drive the transcription of pssY and pssP2. In this case, the level of β-galactosidase activity was significantly higher than in the control in the case of cultures grown in 79CA and M1 media (Figure 3). For comparison, the strong promoter of the pssO gene [32] showed ~20-fold increase in β-galactosidase activity in comparison with pMP220 regardless of the medium used (Figure 3). The promoter of the pssP gene [22], revealed significant increase in activity in case of cultures grown in TY and 79CA medium (Figure 3).

According to the results obtained, it is proposed that pssP2 transcription is most probably driven by a medium active promoter that precedes pssY gene. In the light of the above data, lack of the strong RBS upstream of the pssP2 ORF, the presence of several rare Arg codons in the 5’-end and the data for other genes implicated in the Wzx/Wzy-dependent pathway, the products of which are involved in polysaccharide polymerization.
and transport, it is predicted that the Psp2 protein may not be abundant in RtTA1 cells.

**Psp2 is involved in EPS synthesis**

To investigate the function of *pssP2* and verify its involvement in polysaccharide synthesis, a mutant disrupted in the *pssP2* open reading frame through the integration of the plasmid was constructed. To this end, the integration plasmid pKP2 carrying an internal fragment of *pssP2* ([Figure 2B](#fig2){ref}.) was introduced into the RtTA1 wild type and its integration was forced by an antibiotic selection. The resulting *pssP2::pKP2* mutant was checked for the type of genomic rearrangements through PCR (data not shown).

The results showed that the mutant encoded a shorter variant of Psp2 (N-terminal 433 aa and the 3’-end of *pssP2* was under control of the *plac* promoter. The localization of the promoter ensured that the genes downstream of *pssP2* would be transcribed and the phenotype of the mutant would not be result of polar effects ([Figure 2C](#fig2){ref})).

To complement the mutant phenotype, the *pssP2*-overexpressing plasmid pQBP2his was introduced into the *pssP2::pKP2* strain. The expression plasmid pQBP2his ensured production of His6-Psp2 protein; however, the recombinant protein was poorly detectable when the expression construct was introduced into the *pssP2::pKP2* cells by Western blotting with anti-His antibodies had a molecular mass of ~65 kDa (Figure 4). The presence of the protein was concomitantly checked in *pssP2::pKP2* cells by Western blotting with anti-His antibodies gave no detectable signal, showing that Psp2 might not be phosphorylated, or at least phosphorylation is not detectable with the chosen method (data not shown).

The *pssP2::pKP2* mutant and its complemented derivative *pssP2::pKP2* were subjected to analysis of symbiotic performance. The results were compared with the wild type strain ([Figure 5](#fig5){ref}). The plants

### Table 3. Summary of the highest scoring results from the HHpred search of the PssP2 protein against the PDB database.

| Match | Organism                      | Probability/e-value | Match region in query PssP2 (aa) | Match region in found template (aa) | Secondary structure score | PDB identifier |
|-------|-------------------------------|---------------------|----------------------------------|-------------------------------------|---------------------------|----------------|
| Tyrosine-protein kinase Etk | *Escherichia coli*          | 100.0/2.1e-38       | 328–586                          | 13–282 (299)                      | 26.7                      | 3COI          |
| Tyrosine-protein kinase Wzc | *Escherichia coli*          | 100.0/1.7e-38       | 335–586                          | 12–270 (286)                      | 25.6                      | 3LA6          |
| Tyrosine-protein kinase CapA (C-terminal fragment) | *Staphylococcus aureus* | 100.0/7.6e-36 | 337–587                          | 13–258 (269)                      | 27.8                      | 4JMP          |
| Cell division inhibitor ATPase MinD | *Pyrococcus furiosus* | 99.9/2.2e-23       | 408–585                          | 2–178 (237)                       | 17.6                      | 1G3Q          |
| Bacterial cell division regulator MinD | *Archaeglobus fulgidus* | 99.9/8.1e-23       | 408–584                          | 2–176 (263)                       | 18.8                      | 1HYQ          |
| Chimeric WzzB chain length determinant protein (periplasmic domain) | *Shigella flexneri* | 97.8/0.001          | 60–312                            | 7–248 (248)                       | 16.8                      | 4E29          |
| Lipopolysaccharide biosynthesis protein WzzE | *Escherichia coli* | 97.3/0.0056         | 63–309                            | 1–265 (265)                       | 15.5                      | 3BB0          |
| Bacterial polysaccharide co-polymerase FepE | *Escherichia coli* | 97.0/0.033          | 62–305                            | 12–279 (280)                      | 17.4                      | 3BBM          |

Figure 3. Transcriptional activity of the predicted *pssP2* and *pssY* promoters, as determined by measuring β-galactosidase activity in *R. leguminosarum bv. trifoli* TA1. The strains carrying a plasmid with appropriate promoter fusion were cultured in TY (orange bars), 79CA (light grey) or M1 (dark grey) medium. Values are the means ± standard errors (extended bars) of at least four independent assays and are expressed in Miller units. The bars labeled with asterisks represent β-galactosidase activity values which are significantly different from the empty pMP220 vector control at p<0.05. The results were compared within groups for the three different media. The original names of the constructs for *pssO* and *pssP* genes were changed to avoid confusion only for the sake of data presentation: pMP-P stands for the original pMP2P [22] and pMP-O stands for the pMPO1 [38].

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inoculated with the pssP2::pKP2 mutant gave a significantly better yield of shoot mass than those inoculated with the RtTA1 wild type and the complemented mutant (Figure 5). The pssP2::pKP2 mutant was affected in EPS production as it produced slightly more overall EPS than the wild type, but with an enrichment of LMW fractions (Figure 6C). However, the HMW fractions produced by the mutant had higher molecular masses than in RtTA1 (Figure 6D-i). The molar ratio of glucose/ glucuronic acid/galactose in the EPSs from the RtTA1, the mutants and the complemented strain was 5:2:1, which is characteristic of EPS of R. leguminosarum bv. trifolii (data not shown). It was shown that the quality and quantity of surface polysaccharides may influence autoaggregation of rhizobia.

Figure 4. Western immunoblot analysis with anti-His antibodies of subcellular localisation of His6-PssP2 protein. Fractions analysed contained soluble proteins (SOL) and membrane proteins (TM) of the pssP2::pKP2 mutant and the complemented strain. doi:10.1371/journal.pone.0109106.g004

Figure 5. Symbiotic capabilities of the pssP2::pKP2 mutant and its complemented derivative compared with the wild type RtTA1 strain in a standard plant test. The mean values with standard error presented in the graph result from averaging the nodule number (pink bars), wet masses of plant shoots (mg/plant; green bars) and roots (mg/plant; brown bars) of 20 clover plants. The bars labeled with the same letters represent values which are not significantly different at p<0.05, while various letters represent values which are significantly different at p<0.05. Control plants were not inoculated with bacteria. doi:10.1371/journal.pone.0109106.g005

PssP2 interacts with PssT, PssP and PssC

In light of the changes in the quantity and quality of EPS in RtTA1 derivative producing shorter variant of the PssP2 protein, interaction analyses were undertaken for PssP2 and some of the thus characterized Pss proteins involved in EPS synthesis and transport. To this end, we employed a bacterial two-hybrid system [62]. Plasmids carrying pssT, pssP, and pssL genes were constructed previously [30]. In this work, plasmids encoding T18- or T25-fused pssP2, pssA, and pssC genes were constructed. The E. coli DHM1 cya reporter strain was sequentially transformed with all plasmids expressing fusion proteins. Positive clones representing interacting proteins (blue coloring of colonies and significantly higher levels of β-galactosidase activity than in the control) were obtained for the following pairs: Pss2-PssT (two combinations of fusion plasmids), Pss2-PssP (one combination), Pss2-PssP2 (both combinations; showing its ability to form homo-oligomers), and Pss2-PssC (three combinations) (Figure 8). The results obtained clearly showed that phenotypes resulting from pssP2 mutation and overexpression might have come from the interrelation in which PssP2 is entangled, i.e., homooligomerization that is a characteristic property of polysaccharide co-polymerases and heterotypic interactions with the PssT polymerase, PssP co-polymerase, and at least one previously characterized glycosyltransferase encoded within the Pss-I gene cluster, PssC (Figure 8).

The results obtained for the PssP2-PssP pair were significant only in one combination of fusion plasmids, i.e., pUT18C-PssP/pKT25-PssP2. To exclude a false positive result, a co-purification strategy was employed for pssP2::pKP2(pQBP2his) strain. In such background, PssP2 was His-tagged for purification through affinity chromatography, whereas the PssP remained untagged. Examination of the protein content of the fractions eluted from the affinity column after co-purification indicated that PssP2 and PssP interact with one another (Figure 9A). Control purification from cells not carrying the pssP2-expression plasmid demonstrated that PssP binding to the resin occurred through the interaction with a His-tagged PssP2 (Figure 9B).

Discussion

Genetic control of EPS production in R. leguminosarum bv. trifolii was previously characterized at the molecular level and the functions of genes involved in the process were dissected. The PssT and PssP proteins encoded within the Pss-I region were shown to be involved in polymerization of EPS subunits and production of high- and low-molecular weight fractions of EPS (HMW and
Based on the results from the bacterial two-hybrid analysis, these proteins were proposed to interact with each other [30]. In the case of the PssP protein, it was shown that deletions in different domains caused its inability to form homooligomeric structures, but did not completely diminish the protein's property to interact with PssT [30]. Mutants with shorter PssP variants produced EPS, in which LMW fractions dominated [22]. In the case of PssT, deleting its C-terminal part made the protein more prone to homointeractions, but lack of the same domain made its interactions with PssP impossible [30]. Deleting the C-terminal part of PssT in the RtAH1 mutant resulted in production of EPS with prevalence of HMW fractions [21].

The results obtained in this work indicate functional interconnection between the PssP2 protein encoded within the Pss-II polysaccharide synthesis region with the EPS polymerization system encoded by the genes in the Pss-I region: glycosyltransferase PssC active at the EPS unit assembly step and proteins PssP and PssT involved in polymerization/transport outside the cell.

The mutant with a disrupted pssP2 gene and encoding a protein lacking 153 amino acids from its C-terminal cytoplasmic domain produced more EPS than the wild type strain, and in addition to a quantitative increase, domination of HMW fractions containing chains with molecular masses higher than in the wild type was observed (Figure 6C,D). In line with this was the significant change in the autoaggregation properties of the mutant (Figure 6A,B). The pssP2 integration mutant induced fewer, but all pink (and thus effective, nitrogen-fixing) nodules and the fresh masses of clover plant shoots were higher than in plants infected with the wild type (Figure 5). LMW EPS in S. meliloti was shown to be important for nodule invasion [63,64], and HMW EPS is symbiotically inactive. It was shown that HMW EPS preserve Rhizobium sultae from desiccation [65]. The data concerning the role of HMW EPS in R. leguminosarum is scarce, however certain pieces of data indicate that it may be advantageous to rhizobia during the infection step. The phenotypes of pssP2::pKP2 (this work) and pssP2::pKP2 mutant complemented with the pQBP2 plasmid. The retention times of dextran blue (2 MDa), dextran T550 and dextran T10 (10 kDa) molecular mass markers are indicated in each graph.
Glycosyltransferases involved in synthesis of polysaccharides were shown to form a complex in the membrane [16]. It was proposed that the complex might interact with a flippase and a co-polymerase to regulate the length of produced chains [4]. One of the key players in such an interaction in the case of R. leguminosarum might be the priming glycosyltransferase PsaA [19], as the interaction with the co-polymerase could regulate the flow of subunits to the polymerization centre. Following this, PsaP might bridge Psl and Pst and be involved in HMW polymerization, while Psl2 could serve as a linker between glycosyltransferases and the polymerization centre, but being involved in LMW polymerization. Consequently, the phenotypes observed in the pssP2::pKP2 and its complemented derivative may reflect disturbances in the interactions between the components of the chain-length determination system composed of at least three components: PssP, PssT, and Psl2. It was shown for Xanthomonas campestris and S. flexneri that the level of proteins engaged in polymerization of EPS subunits or O-antigens, respectively, and their protein-protein interactions play an essential role in modulating the polymer chain length [74,76].

The model in which Psl2 and Psl2 could serve opposite roles in determining the EPS length is further supported by their topologies. Psl2 was predicted to have four to five coiled coils. In the case of Psl2, only one periplasmic coiled-coil was predicted with high accuracy and one in the cytoplasmic domain, but with less confidence (Figure 2). The probability of coiled coil formation, location, and the number of the coiled-coil motifs is said to correlate with the degree of polymerization of the polysaccharides. If this were the case for Psl2 and Psl2, Psl2 would be responsible for HMW polymerization, while Psl2 for LMW polymerization. Moreover, the site of the mutation in Psl2 localizes near the secondary coiled-coil in the C-terminal domain of the protein (Figure 2). The C-terminal domain of Psl2 was not important for the interaction with Pst, but indispensable for homoooligomerization [30]. If that had been the case for Psl2, the short variant of the protein in the mutant might have disturbed either homoooligomerization or interactions with other proteins.

Contrary to an assembly model dependent on the stoichiometry of the complex members [77] are the results showing that the chain length determining function of PCP proteins depends on certain amino acid residues [78]. Previously, many mutagenesis studies on residues through Wzz proteins indicated that the function of modal chain length determination may be an overall property of the protein and may not be limited to one particular region [77,79,80]. It was reported that the Wzz level did not correlate with the length of O-antigen chains in P. aeruginosa. The amount of chains correlated with the level of protein production, but the length of O-antigen chains was dependent on a specific amino acid residue in a coiled coil domain [81]. Different amino acids may influence oligomerization and stability of the oligomer [82]. Papolos and Morona [82] noted that chain length was related to the stability of Wzz interactions; they described a positive correlation between dimer stability and the production of longer chain lengths. Changes in the oligomerization ability of mutated proteins may also be the case for the psl2 and psl2 mutants. The Psl2 variants were not able to oligomerize and the mutants produced less LMW EPS [22,30]. Psl2 also oligomerizes (Figure 8), thus secondary coiled-coils disrupted in the mutant might have affected its oligomerization/interaction properties. It seems reasonable that besides specific amino acid residues, any significant distortion of structures of Ps proteins may influence their interaction properties and thus the overall property of polymerization of EPS.
Figure 8. Interactions between PssP2 and other proteins involved in EPS production as analysed through the bacterial two-hybrid system. The graphs present the results of measurements of β-galactosidase activity in E. coli DHM1 carrying respective bait and prey plasmids, the combination of which are presented at the top of panels A and B. Each analysed protein, i.e. PssT, PssP, PssL, PssP2, PssA, and PssC, was encoded as a fusion protein with an adenylate cyclase fragment in vectors pUT18 (N-terminal fusions), pUT18C (C-terminal fusions) (baits), and pKT25 (C-terminal fusions) (preys) differentiated by colour. The β-galactosidase activity was measured in at least 3 independent assays for two colonies (biological repeats) in order to exclude clone-by-clone variation, averaged and expressed in U/mg of bacterial dry weight ± standard error. Positive and negative control values are presented at the bottom of each graph. The controls were: the two interacting leucine zipper domains expressed from pUT18Czip and pKT25zip (positive control), and T18 and T25 cyclase fragments in non-recombinant pUT18(pUT18C) and pKT25 (the negative control). The bars labeled with asterisks represent β-galactosidase activity values which are significantly different from the control at p<0.05.

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Figure 9. Analysis of the interaction between PssP2 and PssP in the pssP2::pKP2 mutant carrying the pQBP2his plasmid by a copurification strategy. Samples of 40 μl of each fraction were separated by SDS-PAGE and visualized. (A) PssP is present in the fraction eluted from the resin, which equals to interaction between PssP and His6-PssP2, that was bound to affinity resin via a His-tag. (B) Negative control verifying that co-purification of PssP is dependent on its interaction with the His-tagged PssP2. L, material loaded to the resin; W, last wash (10 resin volumes); E, elution (1 resin volume). Western blots for each protein are shown below the corresponding gels.

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Several mutated Wzz proteins were undetectable via Western blotting but still produced a regulated chain length [92]. PCP proteins appear to be expressed at a higher level than Wzy polymerases, nevertheless still low. The promoter identified upstream pssP2 is weak and the pssP2 transcription is probably driven by a promoter preceding pssP with the medium activity comparable with the promoter of pssD [93]. pssP2 lacks a strong RBS and has several rare codons in the 5′-end that may further support the low abundance of PssP2 in the RtTA1. It was shown Wzz1 responsible for LMM polymers in P. aeruginosa could complement the phenotype even with the uninduced expression, while Wzz2 (HMW polymers) required induced expression for complementation [14]. The two proteins: PssP and PssP2 may have significantly different abundances in the cell, which would correlate with their functions and the possibly different mode of interaction with PssT. Data concerning promoter activity correlate with the phenotypes of pssP2 and pssP mutants. The pssP2-pKP2 mutant was complemented via uninduced expression, which suggests that the level of protein produced without induction (Figure 4) was sufficient for the cell to restore the function. The pssP null mutant was not complemented and the reason for failure might have been the uninduced expression of pssP used for complementation [22].

The C-terminal cytoplasmic domain of PssP is characterized by the presence of an ATP-binding cassette domain. However, no tyrosine-rich motif, a hypothetic target for the phosphorylating/dephosphorylating activity, is present in this domain, excluding autophosphorylating activity similar to that of S. mitilotti ExoP [83] and E. coli Wzc [84]. PssP2 is also similar to bacterial kinases involved in polysaccharide production, but it is devoid of any specific motifs and appeared not to be phosphorylated. Dissection of the functions of other genes in the Pss-II regions seems to be reasonable to clarify the functional importance of abundance of homologues implicated in polysaccharide synthesis. It cannot be excluded that the genes in the Pss-II region are important for modifications of the EPS HMW:LMW ratio in the plant tissue or under unconsidered environmental conditions.

Supporting Information

Figure S1 Multiple sequence alignment between the PssP2 protein and the proteins mentioned in Table 2. The order of sequences in the alignment is: PssP2 R. leguminosarum bv. trifolii TA1 (ABD36650) (marked with red frame), ExoP Sinorhizobium meliloti (P35698), Wzc Escherichia coli (P76387), CpsD Streptococcus agalactiae (Q3K0T0), Ptk Acinetobacter johnsonii (O52788), Etk Escherichia coli (P58764) and Psp Rhizobium leguminosarum bv. trifolii TA1 (ABD47116). Alignment was performed with the MAFFT tool and visualized with Alignment Viewer (http://toolkit.tuebingen.mpg.de/alignv). Coloring of the alignment is based on the biochemical properties of the amino acids, thus the same color covers both identical and similar amino acids (if applicable).

Author Contributions

Conceived and designed the experiments: MM. Performed the experiments: MM PM JK. Analyzed the data: MM JK. Contributed reagents/materials/analysis tools: MM JK. Wrote the paper: MM PM JK AS.

References

1. Fraysse N, Couderc F, Poinsot V (2003) Surface polysaccharide involvement in establishing the rhizobium-legume symbiosis. Eur J Biochem 270: 1365–1380.
2. Skorupska A, Janczarek M, Marczak M, Mazur A, Król JE, Marczak M (2003) Surface polysaccharide involvement in establishing the rhizobium-legume symbiosis. Eur J Biochem 270: 1365–1380.
3. Taylor VL, Udaskin ML, Islam ST, Lam JS (2013) The D3 bacteriophage is related to various metabolic pathways. Res Microbiol 184: 1–10.
4. Collins RF, Beis K, Dong C, Botting CH, McDonnell C, et al. (2007) The 3D structure of a periplasm-spanning platform required for assembly of group 1 capsular polysaccharides in Escherichia coli. Proc Natl Acad Sci 104: 2390–2395.
5. Kos V, Whitfield C (2010) A Membrane-located glycosyltransferase complex required for biosynthesis of the D-galactan and lipopolysaccharide O-antigen in Klebsiella pneumoniae. J Biol Chem 285: 19668–19677.
6. Collins RF, Beis K, Clarke BR, Morris RJ, Whitfield C (2014) Lipopolysaccharide O-antigen size distribution is determined by a chain extension complex of variable stoichiometry in Escherichia coli O8a. Proc Natl Acad Sci 111: 6407–6412.
7. Janke J, Thiel DE, Johnson J, Wrighton MK, Whitfield C (2014) Protein family promiscuity. Microbiol Mol Biol Rev 64: 180–201.
8. Nickerson NN, Mainprize IL, Hampton L, Jones ML, Naismith JH, et al. (2014) Pseudomonas aeruginosa chain-length regulation in Pseudomonas aeruginosa PA01. Sci Rep 3:3441.
9. Drummelsmith J, Whitfield C (2000) Wzx translocase and the corresponding polymerase and chain length regulator ExoP characterization of pseudomonas aeruginosa PA01. Sci Rep 3:3441.
Pollock TJ, van Workum WA, Thorne L, Mikolajczak MJ, Yamazaki M, et al. (1998) Assignment of biochemical functions to glycosyl transferase genes which are essential for biosynthesis of exopolysaccharides in Sphingomonas strain S80 and Rhizobium leguminosarum. J Bacteriol 180: 386–393.

Guerrero N, Karunamuni VN, Djeujsje VI, Joaquina TV, Rolle BG (2000) Elevated levels of synthesis of over 20 proteins results after mutation of the Rhizobium leguminosarum exopolysaccharide synthesis gene pssA. J Bacteriol 182: 4521–4532.

Morona R, Van Den Bosch L, Daniel C (2000) Evaluation of Wzz/MPA1/MPA2 proteins based on the presence of coiled-coil regions. Microbiology 146: 1–4.

Kalynsky S, Valvano MA, Cygler M (2012) Polysaccharide co-polymers: the enzymatic conductors of the O-antigen assembly orchestra. Post Eng Des Sci 25: 797–802.

Marczak M, Dziwójczyńska M, Skorupka A (2013) Homos- and heterotopic interactions between Psi proteins involved in the exopolysaccharide transport in Rhizobium leguminosarum bv. trifolii. Biochem Biophys Res Commun 394: 341–359.

Król JE, Mazur A, Marczak A, Skorupka A (2007) Syntenic arrangements of the surface polysaccharide biosynthesis genes in Rhizobium leguminosarum. Genomics 89: 237–247.

Sandgren J, Frisch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Vincent JM (1971) Serological properties of the root-nodule bacteria. I. Strains of Rhizobium meliloti. Proc. Loc. Soc. NY 66: 145–154.

Kaczorowski B, Biegańska MA, Olsza C, Józefiak G (1999) New mobilizable vectors suitable for gene replacement in gram-negative bacteria and their use in mapping of the 3' end of the Xanthomonas campestris pv. campestris gum operon. Appl Environ Microbiol 65: 278–282.

Sporns HP, Okáli A, Jöfflein CA, Feis E, Lügtenberg BJ (1987) Promoters in the nodulation region of the Rhizobium leguminosarum Sym plasmid pRL-JP. Plant Mol Biol 9: 27–39.

Kovach ME, Elzer PH, Hill DS, Robertson GT, Farris MA, et al. (1995) Four new GASP primers: the broad-host-range class of bacteriophage vectors. Applied Microbiol 26: 1784–791.

Simon R, Priefer U, Hülser A (1983) A broad host range mobilization system for in vitro genetic engineering: transposon mutagenesis in gram-negative bacteria. BioTechnology 1: 78–84.

Wieloo J, Mazzur A, Król JE, Marczak M, Skorupka A (2004) Environmental modulation of the potNTP gene expression in Rhizobium leguminosarum bv. trifolii. Curr Microbiol 50: 201–207.

Starzynska A, Biegańska MA, Urazhk-Innokentieva T, van Lummersen (1995) Two classes of nodules induced on Trifolium pratense by mutants of Rhizobium leguminosarum bv. trifolii deficient in exopolysaccharide production. J Plant Physiol 147: 93–100.

Yasar S (2005) Spectrophotometric determination of hexose and peptone amounts by artificial neural network calibration and its use in wood analysis. Acta Chim Slov 52: 435–439.

Kutkowska J, Turska-Szewczak A, Janczarek M, Pahurz R, Kaminska T, et al. (2011) Biogefical activity of lipopolysaccharides of the exopolysaccharide-deficient mutant R1210 derived from Rhizobium leguminosarum bv. trifolii strain TAI Biochemistry (Moscow), 76: 840–850.

Apicella MA (2000) Isolation and characterization of lipopolysaccharides. Methods Mol Biol 153: 3–15.

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

Karimova G, Pidoux J, Ullmann A, Ladant D (1998) A bacterial two-hybrid system based on a reconstituted signal transduction pathway. Proc Natl Acad Sci USA 95: 5752–5756.

Batisi I, Lara JC, Leigh JA (1992) Specific oligosaccharide form of the Rhizobium meliloti exopolysaccharide promotes nodules invasion in alfalfa. Proc Natl Acad Sci USA 89: 5626–5630.

Pelock BJ, Cheng HP, Walker GC (2000) Allalfa root nodule invasion efficiency is dependent on Sinorhizobium meliloti polysaccharides. J Bacteriol 182: 4310–4318.

Grazzoni R, Carpigna M-A, Costacurta, F, Bencardino A, Poizot V (2013) Relevance of fucose-rich extracellular polysaccharides produced by Rhizobium vallare strains nodulating Helianthus annuus L. Legumes. Curr Environ Microbiol 79: 1764–1776.

Van Den Bosch L, Manning PA, Morona R (1997) Regulation of O-antigen chain length is required for Shigella flexneri virulence. Mol Microbiol 23: 765–775.

Kinz E, Schar F, DiGiandomenico A, Goldberg JB (2008) Lipopolysaccharide O-antigen chain length regulation in Pseudomonas aeruginosa serogroup O11 strain PA103. J Bacteriol 190: 2709–2716.

Murray GL, Attridge SR, Morona R (2003) Regulation of Salmonella typhimurium lipopolysaccharide O antigen chain length is required for virulence; identification of FepE as a second Wzz. Mol Microbiol 47: 1395–1406.

Murray GL, Attridge SR, Morona R (2005) Inducible serum resistance in Salmonella typhimurium and plasmid-encoded lipopolysaccharide genes on invasion and serum resistance. Mol Microbiol 24: 699–706.

Gallardo E, M. Perez J, Ancillo D, Cukic J, Orusco J, et al. (2012) Relevance of the S. flexneri O-25 polysaccharide as an immobilizer. Microbiology 22: 739–741.

Morona R, van den Bosch L, Manning PA (1995) Molecular, genetic and topological characterization of O-antigen chain length regulation in Shigella flexneri. Microbiology 141: 1039–1048.

Stevenon G, Andrianegagouao K, Hobbs M, Reeves PR (1996) Organization of the Escherichia coli K-12 gene cluster responsible for production of the extracellular polysaccharide colanic acid. J Bacteriol 178: 4885–4893.

Carter JA, Jiminez JC, Zaldívar M, Alvarez SA, Marolda CL, et al. (2009) The cellular level of O-antigen polymers Wzz, Wzz2, and Wzz3 is dependent on the transcriptional regulator OmpC. Mol Microbiol 73: 658–669.

Galván EM, Ielmini MV, Patel YN, Bianco MI, Franceschini EA, et al. (2013) Biological activity of (lipo)polysaccharides of the exopolysaccharide gene cluster EPS Chain Length Determinant in the Salmonella enterica serovar Typhimurium strain PA103. J Bacteriol 195: 2727–2737.

Bouzida M, Shaker AE, Karam M, El Rifi FA, El Rhachi A, et al. (2000) Phosphorylation of Wzc, a tyrosine autokinase, is essential for assembly of the broad-host-range vector pBRIMCs, carrying different antibiotic-resistance cassettes. Gene 166: 175–176.

Thompson J, Higgiin D, Gibson T (1994) CLUSTALW: improved the Clustal alignment search tool. J Mol Biol 215: 403–410.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215: 403–410.

Lupas A, Van Dyke M, Stock J (1993) COILS/PCOILS The program then predicts coiled-coil regions. Science 252: 1162–1164.

Blel N, Gammanolo S, Bruнак S (1999) Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. J Mol Biol 294: 1351–1359.

Tran EN, Morona R (2013) Residues located inside the outer membrane polysaccharide export protein GumB. Glycobiology 23: 259–272.

Kool L, Krogh A, Sonnhammer ELL (2004) Phobius: A Combined Transmembrane Topology and Signal Peptide Prediction Method. J Mol Biol 338: 1027–1036.
Kintz EN, Goldberg JB (2011) Site-directed mutagenesis reveals key residue for O antigen chain length regulation and protein stability in *Pseudomonas aeruginosa Wzz2*. J Biol Chem 286: 44277–44284.

Papadopoulos M, Morona R (2010) Mutagenesis and chemical cross-linking suggest that Wzz dimer stability and oligomerization affect lipopolysaccharide O-antigen modal chain length control. J Bacteriol 192: 3385–3393.

Niemeyer D, Becker A (2001) The molecular weight distribution of succinoglycan produced by *Sinorhizobium meliloti* is influenced by specific tyrosine phosphorylation and ATPase activity of the cytoplasmic domain of the ExoP protein. J Bacteriol 183: 5163–5170.

Grangeasse C, Doublet P, Cozzone AJ (2002) Tyrosine phosphorylation of protein kinase Wzc from *Escherichia coli* K12 occurs through a two-step process. J Biol Chem 277: 7127–7135.

Chakravorty AK, Zurkowski W, Shine J, Rolfe BGJ (1982) Symbiotic nitrogen fixation: molecular cloning of *Rhizobium* genes involved in exopolysaccharide synthesis and effective nodulation. J Mol Appl Genet 1: 585–596.

Glucksmann MA, Reuber TL, Walker GC (1993) Family of glycosyl transferases needed for the synthesis of succinoglycan by *Rhizobium meliloti*. J Bacteriol 175: 7033–7044.

Yamamoto S, Miyake K, Koike Y, Watanabe M, Machida Y, et al. (1999) Molecular characterization of type-specific capsular polysaccharide biosynthesis genes of *Streptococcus agalactiae* type Ia. J Bacteriol 181: 5176–5184.

Grangeasse C, Doublet P, Vaganay E, Vincent C, Delage G, et al. (1997) Characterization of a bacterial gene encoding an autophosphorylating protein tyrosine kinase. Gene 204: 259–265.

Ilan O, Bloch Y, Frankel G, Ullrich H, Geider K, et al. (1999) Protein tyrosine kinases in bacterial pathogens are associated with virulence and production of exopolysaccharide. EMBO J 18: 3241–3248.

Robertson BK, Aman P, Darvill AG, McNeil M, Albersheim P (1981) Host-symbiont interactions. V. The structure of acidic extracellular polysaccharides secreted by *Rhizobium leguminosarum* and *Rhizobium trifolii*. Plant Physiol 67: 389–400.