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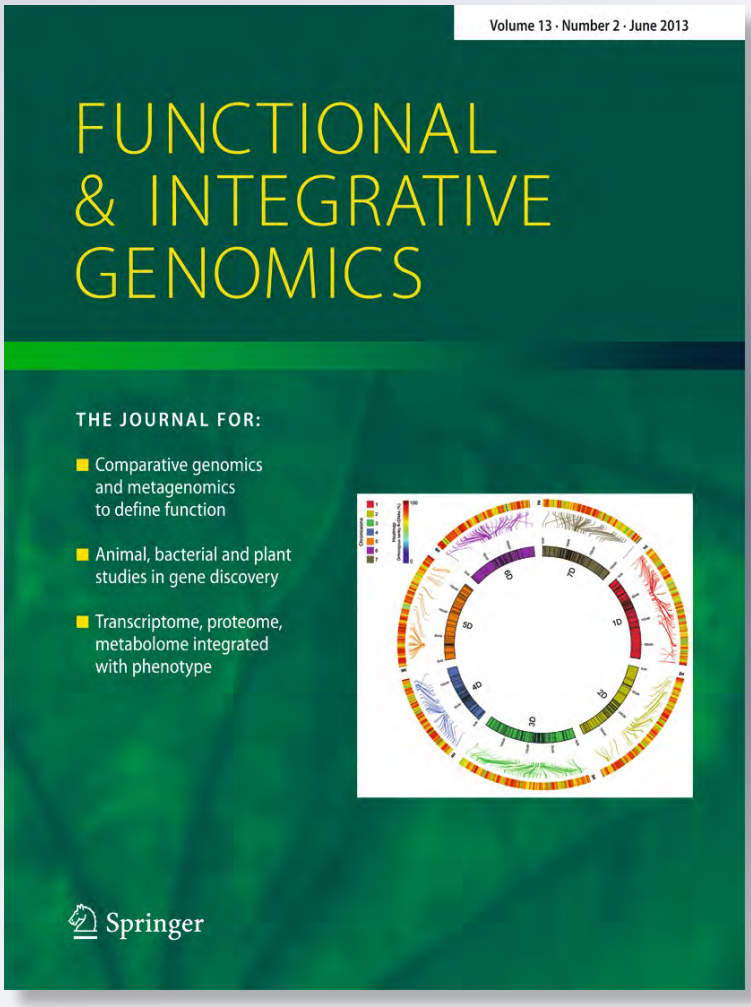
Functional & Integrative Genomics

ISSN 1438-793X
Volume 13
Number 2

Funct Integr Genomics (2013)
13:275-283
DOI 10.1007/s10142-013-0322-7



Springer

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ORIGINAL PAPER

Fast induction of biosynthetic polysaccharide genes *lpxA*, *lpxE*, and *rkpI* of *Rhizobium* sp. strain PRF 81 by common bean seed exudates is indicative of a key role in symbiosis

Luciana Ruano Oliveira · Elisete Pains Rodrigues · Francismar Corrêa Marcelino-Guimarães · André Luiz Martinez Oliveira · Mariangela Hungria

Received: 30 October 2012 / Revised: 15 February 2013 / Accepted: 1 April 2013 / Published online: 8 May 2013

Abstract Rhizobial surface polysaccharides (SPS) are, together with nodulation (Nod) factors, recognized as key molecules for establishment of rhizobia–legume symbiosis. In *Rhizobium tropici*, an important nitrogen-fixing symbiont of common bean (*Phaseolus vulgaris* L.), molecular structures and symbiotic roles of the SPS are poorly understood. In this study, *Rhizobium* sp. strain PRF 81 genes, belonging to the *R. tropici* group, were investigated: *lpxA* and *lpxE*, involved in biosynthesis and modification of the lipid-A anchor of lipopolysaccharide (LPS), and *rkpI*, involved in synthesis of a lipid carrier required for production of capsular polysaccharides (KPS). Reverse transcription quantitative PCR (RT-qPCR) analysis revealed, for the first time, that inducers released from common bean seeds strongly stimulated expression of all three SPS genes. When PRF 81 cells were grown for 48 h in the presence of seed exudates, twofold increases ($p<0.05$) in the transcription levels of *lpxE*, *lpxA*, and *rkpI* genes were observed. However, higher increases ($p<0.05$) in transcription rates, about 50-fold for *lpxE* and about 30-fold for *lpxA* and *rkpI*, were observed after only 5 min of incubation with common bean seed exudates. Evolutionary analyses revealed that *lpxA* and *lpxE* of PRF81 and of the type strain of *R. tropici* CIAT899^{T}clustered with orthologous *Rhizobium radiobacter* and were more related to *R. etli* and *Rhizobium leguminosarum*, while *rkpI* was closer to the *Sinorhizobium* sp. group. Upregulation of *lpxE*, *lpxA*, and *rkpI* genes suggests that seed exudates can modulate production of SPS of *Rhizobium* sp. PRF81, leading to cell wall changes necessary for symbiosis establishment.

Luciana Ruano Oliveira and Elisete Pains Rodrigues contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s10142-013-0322-7) contains supplementary material, which is available to authorized users.

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Keywords Biological nitrogen fixation · K-Antigen polysaccharide · LPS · *Rhizobium tropici* · RT-qPCR · Symbiosis

Introduction

Nitrogen-fixing soil bacteria, collectively called rhizobia, comprise diverse genera, as *Bradyrhizobium*, *Mesorhizobium*, *Azorhizobium*, *Sinorhizobium*, and *Rhizobium*. Rhizobia are capable of colonizing and inducing formation of nodules on roots of leguminous plants in host-specific symbioses (Fauvart and Michiels 2008). In the nodule, rhizobial cells, in the form of bacteroids, provide nitrogen to the host plant, promoting growth at low cost, economically and environmentally, especially under nitrogen-limiting conditions.

Nodule development in rhizobia–legume symbiosis involves several steps that are determined by genes in both symbiotic partners. Early in development of the symbiosis, bacterial and plant genes are activated by a signaling cascade exchange that starts with release of seed and root exudates that induce expression of nodulation (Nod) genes in the rhizobia. In response, the rhizobia synthesize and excrete lipochitooligosaccharide signals (Nod factors) that elicit host-plant-specific responses, such as root hair deformation, initiation of nodule meristems, and induction of early nodulin genes, leading to nodule occupancy and development (Cooper 2007; Ferguson et al. 2010; Hungria et al. 1992; Schultze and Kondorosi 1996).

In addition to Nod factors, it is recognized that successful establishment of symbioses involves rhizobial surface polysaccharides (SPS), with important roles at several stages of the symbiosis development (Becker et al. 2005; Cooper 2007; D'Haeze and Holsters 2004; Downie 2010; Fraysse et al. 2003). SPS comprise distinct molecules, such as exopolysaccharides (EPS), lipopolysaccharides (LPS), capsular polysaccharides (KPS or K-antigens), and cyclic β-glucans. EPS and KPS play major roles in mutual recognition of symbiotic partners and infection process, whereas LPS exhibit specific active roles in later stages of the Nod process, such as penetration of cortical cells by infection thread; thereby, they represent key molecules for the Fix^+ phenotype of the rhizobia (Becker et al. 2005; Fraysse et al. 2003).

Unlike majority of cultivated legume species, common bean (*Phaseolus vulgaris* L.) is recognized as a nonselective host, able to form nodules with a large range of *Rhizobium* species, although only in a minority of cases are the symbioses effective in fixing nitrogen (Grange and Hungria 2004; Hungria and Neves 1987; Martinez-Romero 2003; Michiels et al. 1998). Regarding the rhizobia that usually nodulate common bean roots, strains belonging to the *Rhizobium tropici* group seem to be the most suitable species for tropical and subtropical conditions, such as those found in Brazil (Hungria et al. 2000, 2003; Pinto et al. 2007). PRF 81 is a *Rhizobium* sp. strain that belongs to the *R. tropici* group (Ormeño-Orrillo et al. 2012; Ribeiro et al. 2012); it results from a selection program in Brazil and is now commonly used in commercial inoculants, being very tolerant to stressful tropical conditions (Hungria et al. 2000, 2003).

Despite the importance of the *R. tropici–P. vulgaris* symbiosis, molecular determinants of the interaction with strains belonging to the *R. tropici* group are still poorly understood. Availability of complete genome sequences of strains PRF 81 and CIAT 899 (Ormeño-Orrillo et al. 2012) has recently revealed new and interesting information on the genetics of *R. tropici*. Here, we report studies on the expression of *rkpI*, *lpxA*, and *lpxE* genes, which are involved in biosynthesis of KPS and LPS, when induced by exudates from common bean seeds.

Materials and methods

Strains, media, and growth conditions

Rhizobium sp. strain PRF 81 (=SEMIA 4080) was obtained from the Diazotrophic and Plant Growth Promoting Bacteria Culture Collection of Embrapa Soja and transferred to Petri plates containing yeast mannitol medium (Vincent 1970) with Congo red (0.025 %) and incubated at 28 °C for 48 h. To obtain preinoculum, cells were cultured in 10 mL of liquid tryptone–yeast (TY) broth (Beringer 1974) and shaken at 120 rpm until an optical density (OD) of 0.9 at 600 nm was reached.

Assays for induction of polysaccharide genes

In the first assay, 1 mL of the preinoculum was added to 200 mL of TY broth and incubated for 48 h in the presence of exudates from common bean seeds, prepared as previously described (Hungria et al. 1991a). The final concentration of seed exudates was estimated by the OD at 289 nm using the extinction coefficient of naringenin ($\log_\varepsilon$=4.23 at 289 nm) to result in an approximate concentration of 1.5 μM. After 48 h (OD 0.4–0.8) of exposure, the gene expression/transcription was evaluated.

In the second assay, cells were cultured until reaching exponential growth phase (OD 0.4–0.8) and after that, seed exudates, adjusted to an approximate concentration of 1.5 μM, as described for the first assay, were added to the culture broth and gene expression was determined after various periods of exposure: 5 min (T_5min), 15 min (T_{15}min), 1 h (T_1h), 4 h (T_4h), and 8 h (T_8h). Water (the same volume as the seed exudates) was used as the control treatment for gene expression/transcription variation. The assays were conducted in a completely randomized block design with three biological replicates, represented by three independent

experiments, of cultures grown for different times, and each experiment was performed with three replicates.

RNA extraction and primers design

Total RNA from these bacteria was prepared as described before (Farrell Jr 1998), and a detailed description of the procedure has been given before (Oliveira et al. 2010). The first strand of cDNA was obtained using 1.5 μg of RNA and the TaqMan® Reverse Transcription Reagents kit (Applied Biosystems, Foster, CA, USA), following the manufacturer's instructions.

Primers were designed using PrimerExpress3.0 (Applied Biosystems) targeting an amplicon size of 50–150 bp. The partial genome of strain PRF 81 (Pinto et al. 2009) was used to design primers specific for *lpxA*, *lpxE*, and *rkpI* genes. The primers used are listed in Table 1.

RT-qPCR and statistical analysis

Each biological sample was amplified in three replicates with primers (300 nM) for the *lpxA*, *lpxE*, and *rkpI* genes and also with primers (200 nM) for the 16S rRNA gene (Table 1), used as the endogenous control, and the SYBR Green Master Mix Kit (Applied Biosystems), in a final volume of 25 μL. Reactions were performed on a 7500 Reverse transcription quantitative PCR (RT-qPCR) thermocycler (Applied Biosystems), following the manufacturer's instructions. After the initial steps at 50 °C for 2 min (uracil-N-glycosylase activity) and at 95 °C for 10 min (activation of the AmpliTaq Gold polymerase), a two-step program of 95 °C for 15 s and 62 °C for 1 min was conducted for 40 cycles. Dissociation curves were obtained to guarantee the absence of unspecific amplifications. The data were collected in the last phase (extension phase), and the Relative Expression Software Tool—Multiple Condition Solver (REST-MCS©, version 2) was used to calculate the relative expression of target-gene mRNA in RT-qPCR using the Pair Wise Fixed Reallocation Randomization Test© (Pfaffl et al. 2002). The mathematical model used was based on the mean crossing point (CP or Ct, cycle threshold) deviation between the sample and control group of the target gene, normalized by the mean crossing point deviation of the endogenous control gene. Specific amplification efficiency was included in the correction of the quantification ratio. The formula $E = \left[10^{1/\text{slope}}\right] - 1$ was used to calculate the reaction efficiency. The calibration curve was established by the Ct and the log of the cDNA dilutions. The relative quantification was expressed as means ± standard error (SE) from three independent experiments for each set of conditions tested. Statistical significance was determined using the REST-MCS© test with 2,000 randomization iterations and p values <0.05 were considered significant.

Table 1 Sequences of the primers used in the RT-qPCR and sizes of the PCR products obtained

Gene/ORF ID	Primers sequence	Amplicon size
lpxA/RQ82527	F 5′ TCCAAGGTTGTGCTGCATGA 3′ R 5′ GTCGTGCGGCCGGTAAC 3′	65 bp
lpxE/RQ76436	F 5′ GGCGCTGCCCTGAAGAA 3′ R 5′ GCCGAAATCGGTCAGCATT 3′	66 bp
rkpI/RQ17072	F 5′ TGCCGTGGCCATTGC 3′ R 5′ AAGCGACAGACCGGTTCACT 3′	66 bp
16SrRNA	F 5′ CAAGGCGACGATCCATAGCT 3′ R 5′ AGGAGTTTGGGCCGTGTCT 3′	72 bp

Genomic and phylogenetic analyses of *lpxA*, *lpxE*, and *rkpI*

BLAST searches (Altschul et al. 1997) using amino acid sequences of *lpxA*, *lpxE*, and *rkpI* genes from *Rhizobium* sp. strain PRF81 (Ormeño-Orrillo et al. 2012) were performed at the National Center for Biotechnology Information (NCBI) server. Homologous sequences that had at least 60 % of cover and 35 % of identity were selected and corresponding nucleotide sequences were obtained from GenBank database. Unrooted phylogenetic trees were constructed based on ClustalW multiple sequence alignment using the neighbor-joining (NJ) (Saitou and Nei 1987) and maximum composite likelihood methods (Felsenstein 1985; Saitou and Nei 1987; Tamura et al. 2004) using 2,000 bootstrap replicates (Felsenstein 1985). Evolutionary relationships were carried out with MEGA 5 software package (Tamura et al. 2011). Comparative analyses were also performed using RAST (Rapid Annotation using Subsystem Technology) server (Aziz et al. 2008) and the NCBI database.

Results

Expression levels of *lpxA*, *lpxE*, and *rkpI*

In the first assay, in which cells were grown for 48 h in the presence of seed exudates, significant increases, of about twofold ($p<0.05$), were observed in the transcription level of gene *rkpI*, involved in production of KPS as well as of genes *lpxA* and *lpxE*, related to the biosynthesis and modification of LPS (Fig. 1). In addition, in the second assay, we found that after only 5 min of exposure to the seed exudates, the level of transcripts statistically increased ($p<0.05$) by about 50-fold for *lpxE* and about 30-fold for *lpxA* and *rkpI*. Subsequently, *Rhizobium* sp. PRF 81 cells recovered basal levels of transcripts of the three genes (Fig. 1).

The results obtained in our study have then shown that *lpxA*, *lpxE*, and *rkpI* genes of PRF81 were upregulated in the

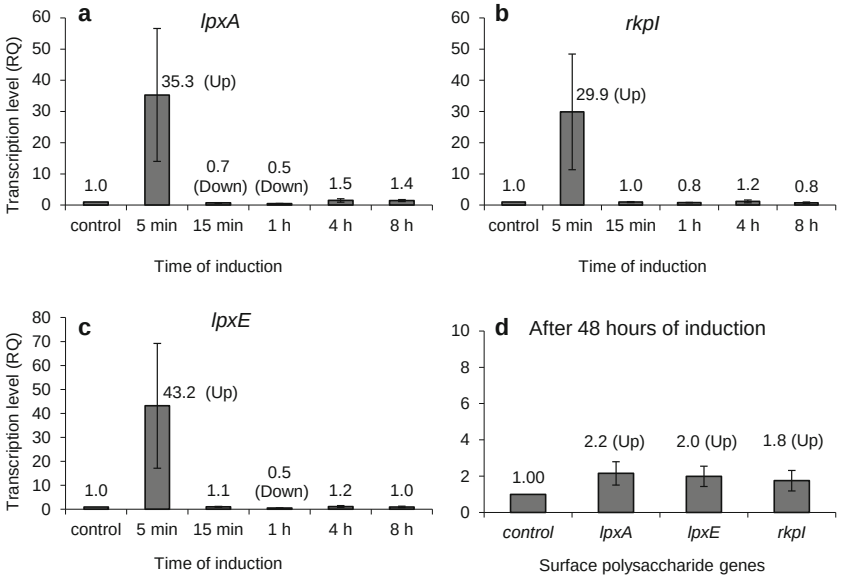

Fig. 1 Expression levels of *lpxA*, *lpxE*, and *rkpI* genes of *Rhizobium tropici* strain PRF 81 after exposure to common bean seed exudates. Exponential phase cells of *R. tropici* were exposed to exudates for various periods of time (**a**, **b**, and **c**) or were grown for 48 h in culture medium containing exudates (**d**). Relative quantification (RQ) was estimated by REST-MCS© software and expressed as means ± standard error (SE) from three independent experiments, each with three replicates. Statistical significance was determined using REST-MCS© test with 2,000 randomization iterations and p values <0.05 were considered significant. The 16S rRNA gene was used as the endogenous control

cells by common bean seed exudates. Therefore, the results demonstrate that these inducers can modulate not only Nod genes but also biosynthesis and modification of SPS of strains belonging to the *R. tropici* group.

Genomic and phylogenetic analyses

The biosynthesis of LPS is initiated by acylation of UDP-*N*-acetylglucosamine, a reaction catalyzed by acyltransferase LpxA, a product of *lpxA* gene. The predicted LpxA (RQ82527) of *Rhizobium* sp. PRF81 (Ormeño-Orrillo et al. 2012) consists of a polypeptide of 271 amino acids, which showed 95 and 86 % identities to LpxA of *Rhizobium* (=*Agrobacterium*) *radiobacter* K84 (YP_002544398.1) and *Rhizobium leguminosarum* bv. trifolii (YP_002975610.1), respectively. Likely, the NJ phylogenetic analysis of *lpxA* homologous (>50 % identity; > 60 % cover) in MEGA 5 showed that *lpxA* of *Rhizobium* sp. PRF81 and of *R. tropici* CIAT899^T are clustered closer to *R. radiobacter*, *R. etli*, and *R. leguminosarum* (Fig. 2). Genomic analyses showed that the *lpxA* gene of PRF 81 is located in the chromosome, clustered with *fabZ*, *lpxD*, and *lpxB* genes, as also described in *Escherichia coli* and other bacterial species (Wang and Quinn 2010). Indeed, an arrangement of seven genes (*ompD-lpxD-fabZ-lpxA-RQ13035-lpxB-gltA*) was observed in PRF 81 and is also conserved between rhizobial species (Fig. 2), such as *R. radiobacter* K84 (NC_011985.1), *R. leguminosarum* bv. trifolii WSM1325 (NC_012850.1), *R. etli* CIAT 652 (NC_010994.1), *Sinorhizobum fredii* HH103 (NC_016812.1), and *Sinorhizobium meliloti* (NC_003047.1). In this cluster, the gene for RQ13035 encodes a conserved hypothetical protein that belongs to a protein superfamily (DUF1009), and in *Caulobacter crescentus*, an ortholog gene named *lpxI* codes an LpxI protein that acts as an alternative UDP-2,3-diacylglucosamine hydrolase in vivo (Metzger IV and Raetz 2010).

The basic structure of LPS may undergo additional modifications in its structure, as the removal of phosphate groups by lipid-A phosphatases LpxE and LpxF is observed in some rhizobia species. The chromosome of *Rhizobium* sp. PRF81 contains an *lpxE* gene (RQ 76436) of 744 bp, which encodes a putative membrane-associated lipid-A 1-phosphatase (LpxE) of 248 amino acids with 79 and 60 % of identities to LpxE of *R. radiobacter* K84 (YP_002546441.1) and of *R. leguminosarum* bv. viciae 3841 (YP_770270.1), respectively. Similar to the observations with *lpxA* (Fig. 2), the NJ dendrogram generated with *lpxE* sequences (>35 % identity; >60 % cover) showed that *lpxE* of *Rhizobium* sp. PRF81 was closer to *lpxE* homologous of CIAT899^T and *R. radiobacter* than of *R. etli* and *R. leguminosarum* (Suppl. fig. 1). Organization of the gene *lpxE* (Suppl. fig. 1) showed to be conserved in CIAT899^T and *R. radiobacter* K84, in which *lpxE* is followed by a gene encoding a putative transcriptional regulator of the ArsR family and by a gene that encodes a 3-oxoacyl-[ACP] reductase (FabG), an enzyme involved in biosynthesis of fatty acid and phospholipids. Also, *lpxE* is preceded by *leuB* and *leuD* genes that encode enzymes involved in biosynthesis of the amino acid leucine. The predicted LpxE amino acid sequence contains a PAP2_lipid-A 1-phosphatase subfamily

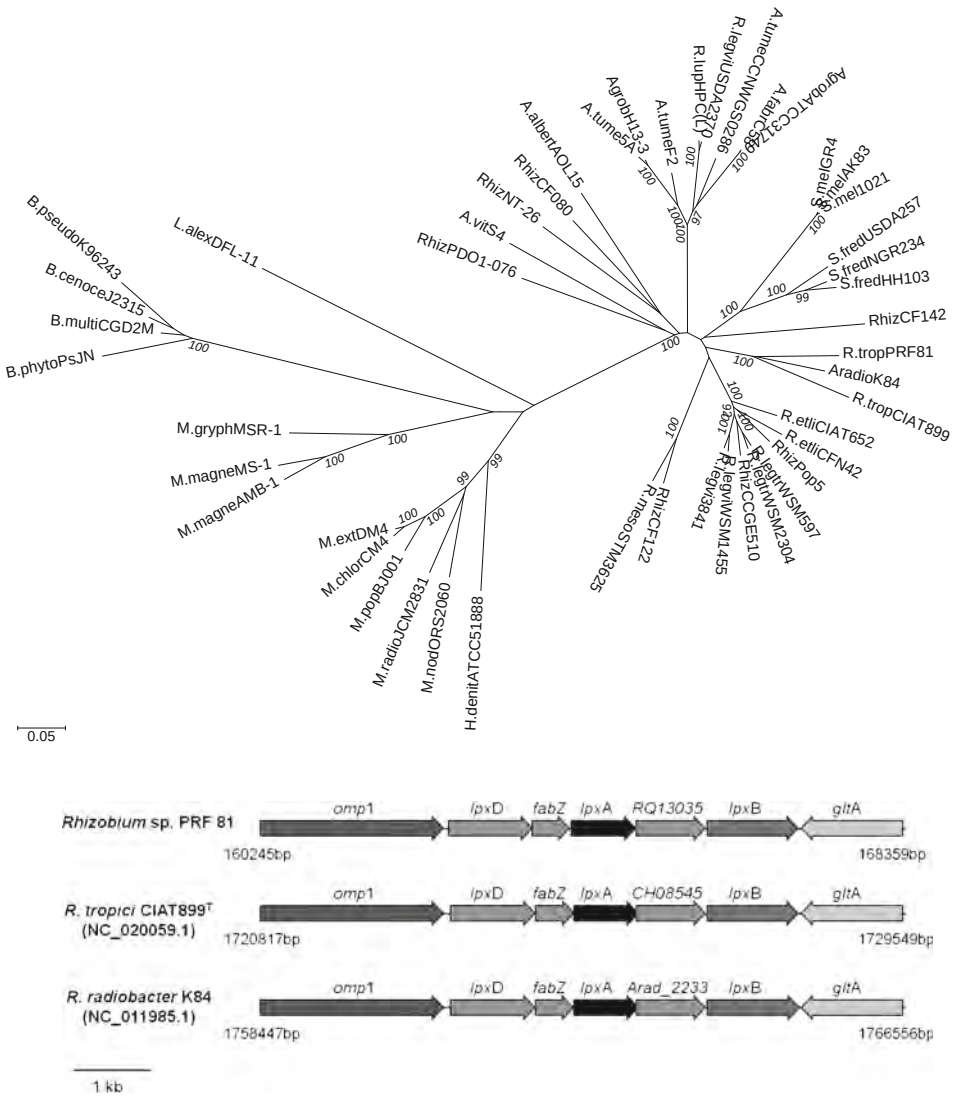

Fig. 2 Unrooted phylogenetic analysis (*top*) and genomic arrangement (*bottom*) of *lpxA* gene from closely related bacteria. Neighbor-joining analysis was based on ClustalW alignment of translated nucleotide sequences (accession numbers are provided as supplementary file) using the maximum composite likelihood method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2,000 replicates) is shown above the branches. Only bootstrapping values >80 are shown. Evolutionary analyses were performed in MEGA 5. (Saitou and Nei 1987; Tamura et al. 2004; Felsenstein 1985; Tamura et al. 2011)

(cd03389; locations 38–222; bit score 164) and six transmembrane helices, as observed in an *R. leguminosarum* LpxE ortholog, which lipid-A 1-phosphatase activity has been confirmed (Karbarz et al. 2003, 2009). In addition to *lpxE*, the PRF 81 genome has an *lpxF* ortholog (RQ 82662) that is predicted to encode a putative lipid-A 4-phosphatase.

In relation to the KPS, the predicted amino acid sequence of the *rkpI* gene of PRF81 (RQ 17072) consists of 531 residues with 63 and 62 % identities to a KPS biosynthesis/export transmembrane protein RkpI of *S. fredii* HH103 (YP_005187553.1) and of *S. meliloti* 1021 (YP_005718746.1), respectively. The NJ dendrogram of *rkpI* homologous (>35 % identity; >60 % cover) showed that *rkpI* of PRF81 and of CIAT899^T was clustered with the *Sinorhizobium* sp. group (Fig. 3). As revealed by partial genome analysis of the *R. tropici* strain PRF 81 (Pinto et al. 2009) and of the whole genomes of *R. tropici* strains CIAT 899^T and of *Rhizobium* sp. PRF 81 (Ormeño-Orrillo et al. 2012), the *rkpI* gene of PRF 81 is located in the *rkp*-1 gene cluster (Fig. 3) required for KPS production, in which the *rkpAGHIJ* genes are arranged similarly to *S. meliloti* 1021 and in *S. fredii* HH103 (Parada et al. 2006), except for the *rkpU* orientation and absence of *kpsF3* gene (Fig. 3).

Discussion

SPS, including EPS, LPS, KPS, and cyclic β-glucans, are key molecules in the establishment of rhizobia–legume symbioses. They are involved in different steps of the Nod process, such as root colonization, host recognition, infection thread formation, inhibition of plant defense responses to allow rhizobia invasion, and in the protection against reactive oxygen species released during the infection process (Becker et al. 2005; Cooper 2007; D'Haeze and Holsters 2004; Fraysse et al. 2003).

Despite extensive studies with various rhizobial species, the precise roles of SPS in the establishment of symbiosis

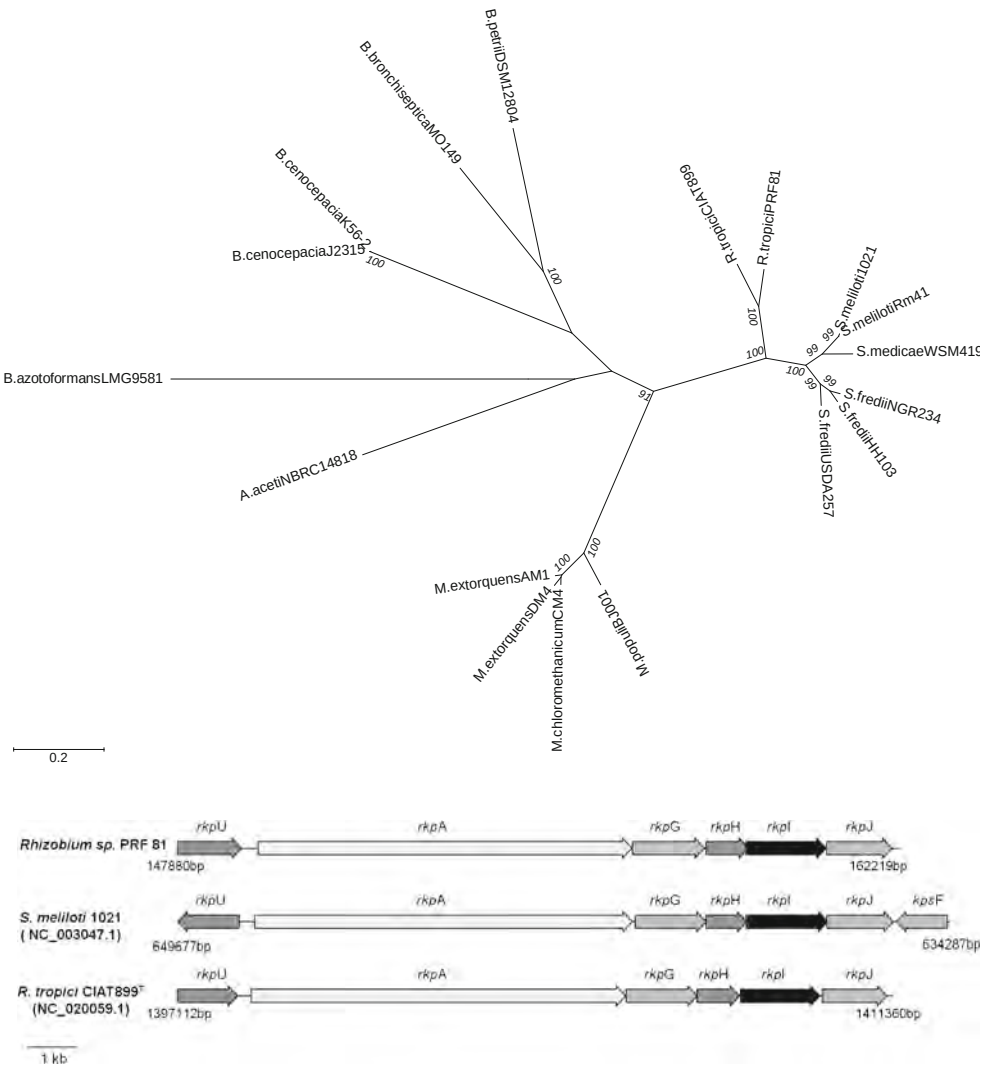

Fig. 3 Unrooted phylogenetic analysis (*top*) and genomic arrangement (*bottom*) of *rkpI* gene from closely related bacteria. Parameters for the analyses were as described for Fig. 2

remain unclear. Likewise, the factors determining the regulation of the production of SPS are also unclear. Regarding *R. tropici*, the molecular structures of the EPS, LPS, and glucans of the type strain CIAT 899^{T} have been described, although the genes and proteins involved in their biosynthesis remain unknown (Gil-Serrano et al. 1990, 1994, 1995, 1993), and there are still no reports on the KPS structure of *R. tropici*. To shed light on these issues, we searched the draft (Pinto et al. 2009) and further the complete (Ormeño-Orrillo et al. 2012) genome of *Rhizobium* sp. strain PRF 81 to identify open reading frames (ORFs) involved in the biosynthesis and modification of LPS and KPS. The transcription of these ORFs was then analyzed by RT-qPCR, after cell induction with exudates from common bean seeds.

Significant increases were observed in the transcription level of the genes *rkpI*, involved in production of KPS, *lpxA* and *lpxE*, related to the biosynthesis and modification of LPS, after growing for 48 h in the presence of seed exudates. However, intriguingly, far greater levels of transcription were observed after only 5 min of exposure to the seed exudates, followed by prompt recovery of the levels of transcripts. In a previous study using RT-qPCR, we found that the transcription of Nod genes *nodC*, *nodG*, and of gene *glgX* (involved in the transformation of glycogen into 1,4-glycan and located immediately after *nodN*) of strain PRF 81 was strongly induced by common bean seed exudates (Oliveira et al. 2010), whereas flavonoid naringenin, a predominant inducer released from common bean roots (Hungria et al. 1991b), provoked much lower responses at equivalent concentrations (Oliveira et al. 2010). Common bean seeds produce a variety of flavonoids acting as *nod* gene inducers (Hungria et al. 1991a); therefore, a combination of flavonoids may be more effective than individually. Here, we confirm that in contact with seed inducers, genes not only for Nod (Oliveira et al. 2010) but also for polysaccharides biosynthesis (Fig. 1) have an extremely fast responses at the transcription level, followed by drastic decreases after a few minutes.

It is known that rhizobial LPS undergo structural modifications in response to the presence of *nod* gene inducers (Broughton et al. 2006; Fraysse et al. 2002; Le Quéré et al. 2006; Reuhs et al. 1994; Simsek et al. 2009). The LPS are found

anchored into the outer leaflet of the external membrane of Gram-negative cells and consist of a lipid-A anchor and a core polysaccharide, which are linked by 3-deoxy-D-manno-oct-2-ulopyranosonic acid (Kdo) residue. The core moiety can carry an O-antigen polysaccharide, responsible for the antigenic property of LPSs (Becker et al. 2005; Fraysse et al. 2003). Production of LPS requires diverse biosynthetic and export enzymes, in addition to modifying enzymes that alter the LPS basic structure in a specific way for each bacterium. In contrast to the modifying enzymes, the enzymes that catalyze early steps in the Kdo–lipid A biosynthetic pathway are present in virtually all Gram-negative bacteria and, in general, are not subjected to regulation (Raetz et al. 2007; Wang and Quinn 2010).

In our study, *lpxA* and *lpxE* genes of PRF81 were upregulated in the cells by seed exudates, indicating that these inducers can modulate the biosynthesis and modification of the LPS of this strain. The biosynthetic pathway of LPS is initiated by fatty acylation of UDP-*N*-acetylglucosamine, a reaction catalyzed by the acyltransferase LpxA (EC:2.3.1.129), which is encoded by the *lpxA* gene (Raetz et al. 2007; Wang and Quinn 2010). As *lpxA* is organized in a cluster together with other genes involved in LPS biosynthesis (Fig. 2), it would be interesting to investigate if seed exudates also regulate the expression of other genes of the *ompD-lpxD-fabZ-lpxA-RQ13035-lpxB-gltA* cluster. It is noteworthy that in a study about the global changes in gene expression in bacteroids and free-living cells of *S. meliloti* 1021, genes *acpXL*, *lpxD*, *fabZ*, and *lpxA* involved in lipid-A synthesis were repressed, whereas *lpsB*, encoding a glycosyltransferase that participates in the biosynthesis of the LPS core was induced in bacteroids (Becker et al. 2004). Studies of differential expression in bacteroids have not been performed yet in strains of the *R. tropici* group; however, the induction of *lpxA* by common bean seed exudates in PRF 81 is indicative that LpxA acyltransferase might be required for cell wall adaptation during development of the symbiosis.

Cell wall adaptation by changes on LPS structure of rhizobial species has been reported during bacteroid differentiation and also in vitro, in response to physiological conditions as well as to the presence of *nod* gene inducers. In *Sinorhizobium* sp. NGR234, it has been suggested that bacterial differentiation into bacteroids is characterized by a reduction of KPS production and an induction of symbiotically active rhamnan-LPS, which would allow a close contact between the microsymbionts and the plant cell membranes (Le Quéré et al. 2006; Simsek et al. 2009). These cell wall changes are modulated by the flavonoid apigenin (Le Quéré et al. 2006; Simsek et al. 2009) and require a *nod*-box transcription regulator (*nodD1*) and Y4gM protein (Simsek et al. 2009). In *R. etli* CE3, common bean seed exudates containing anthocyanins induced changes in the sugar composition of the LPS O-antigen but did not require the *nodD* regulatory protein (Noel et al. 2004; Noel et al. 1996).

Alterations in LPS structure are not restricted to core and O-antigen domain of LPS molecules. In cells of *R. leguminosarum* grown under reduced oxygen levels and acidic conditions, mimicking those within the root nodule, LPS were mostly hydrophobic, with a lipid-A domain enriched with long (3-OH-C18:0) and very long (27-OH-C28:0) fatty acyl chains, suggesting that the expression of the acyltransferases was regulated (possibly involving multiple acyltransferases) by physiological conditions (Kannenberg and Carlson 2001). Although the molecular structure of lipid A of free-living cells of *R. tropici* CIAT899^T is known (Gil-Serrano et al. 1994), structural changes under symbiotic conditions or induced by plant-host exudates have not been reported.

In some bacteria, LPS structure can be modified by additional enzymes, which are not required for survival, but are tightly regulated in the cell and closely related to virulence, helping them to resist cationic antimicrobial peptides (CAMPs) released by the host immune system or to evade recognition by the innate immune receptor TLR4 (Wang and Quinn 2010). For example, in *R. leguminosarum* and *R. etli*, lipid-A molecules lack the 1- and 4-phosphate groups, which are produced by action of lipid-A phosphatases LpxE and LpxF, respectively (Karbarz et al. 2003, 2009; Que et al. 2000). In *R. etli*, LpxE and LpxF single mutants have shown altered lipid-A species containing one or both phosphate groups and an increased sensitivity to polymyxin B; however, all mutants formed nitrogen-fixing nodules in common bean (Ingram et al. 2010). According to these authors, dephosphorylation of lipid-A molecules in *R. etli* is not required for Nod but might play a role in protecting the bacteria from CAMPs, or other immune responses of plants. The presence of *lpxE* and *lpxF* orthologs in PRF 81 genome suggests that the strain synthesizes lipid-A species lacking the usual phosphate groups in the 1 and 4 positions, as observed in *R. etli* and *R. leguminosarum*. In addition, the upregulation of *lpxE* by the host seed exudates indicates that its protein product might be necessary for the establishment of the symbiosis.

LPS was not the only cell SPS affected by seed exudates; the transcript levels of the KPS biosynthetic gene *rkpI* were also notably higher in cells induced with seed exudates (Fig. 1). To our knowledge, there is no information about structure, production, or function of the KPS of strains belonging to the *R. tropici* group. The *rkpI* gene encodes a putative phosphoglycerol transferase (EC 2.7.8.20), possibly involved in the synthesis of a phospholipid carrier of rhizobia K-antigen polysaccharide (Margaret-Oliver et al. 2012; Parada et al. 2006). Studies have shown that rhizobial mutants affected in *rkp* genes of the rkp-1 region are significantly altered in KPS production and in the establishment of an effective symbiosis (Kiss et al. 1997; Parada et al. 2006). Recently, it was shown that mutations in the *rkpA* and *rkpI* genes of *S. fredii* HH103 had significant negative effects on KPS production and in the Nod and nitrogen supply of soybean [*Glycine max* (L.) Merr.] cultivars Williams and Peking (Margaret-Oliver et al. 2012). In this same study, β-

galactosidase assays showed that the presence of the flavonoids genistein or coumestrol had no or only a slight effect in the expression of *rkpA* and *rkpI* genes, although it has been suggested that the transcription of these genes might occur in the bacteroids of soybean nodules (Margaret-Oliver et al. 2012). Similar results have been previously observed for the *rkpG* and *rkpH* genes of the rkp-1 region (Parada et al. 2006).

Since the *rkpI* gene of PRF 81 was upregulated by exudates from common bean seeds, it is probable that the protein coded by this gene might be required for KPS biosynthesis or modification during the symbiosis. Reports of modulation of the production of rhizobial KPS by host-plant-derived compounds show variability of results. In *S. fredii* strain USDA 205, KPS production was altered and had a higher proportion of a secondary 2-*O*-methylmannose-K of the polysaccharide in the cell extracts induced by the flavonoid apigenin, or by soybean root extract (Fraysse et al. 2003; Reuhs et al. 1994). In *S. melilot* strain AK631, alfalfa (*Medicago sativa* L.) root extract increased KPS production, whereas soybean root extract and the flavonoids apigenin, leuteolin, naringenin, and genistein had no apparent effect (Reuhs et al. 1994).

In conclusion, our study has shown notable induction of *lpxA*, *lpxE*, and *rkpI* genes of *Rhizobium* sp. PRF 81 by common bean seed exudates, encouraging further studies of the production and modification of LPS and KPS in cells in vitro and in symbiotic conditions. In addition, mutants defective in KPS and LPS genes will be useful in determining the precise roles of these SPS in the symbioses of common bean with strains of the *R. tropici* group. Finally, it was interesting to observe differences in the evolutionary history of these three genes, as in the analyses of *lpxA* and *lpxE* of PRF 81 and CIAT 899^T, the strains were grouped with orthologous *radiobacter*, being more related to *R. etli* and *R. leguminosarum*, while for the *rkpI* gene being closer to the *Sinorhizobium* sp. group.

Acknowledgments The study was partially supported by the CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil) (Repensa, 557746/2009-4). L. R. Oliveira acknowledges an M.Sc. fellowship (552874/2008-6) and M. Hungria, a research fellowship (300547/2010-2) from CNPq. The authors thank Allan R.J. Eaglesham for help with the manuscript. Manuscript approved for publication by the Editorial Board of Embrapa Soja as manuscript 18/2012.

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