Antonie van Leeuwenhoek (2009) 96:471–486
DOI 10.1007/s10482-009-9362-3

ORIGINAL PAPER

Multiple copies of *rosR* and *pssA* genes enhance exopolysaccharide production, symbiotic competitiveness and clover nodulation in *Rhizobium leguminosarum* bv. *trifolii*

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Received: 23 March 2009 / Accepted: 24 June 2009 / Published online: 9 July 2009

Abstract *Rhizobium leguminosarum* bv. *trifolii* exopolysaccharide (EPS) plays an important role in determining symbiotic competence. The *pssA* gene encoding the first glucosyl-IP-transferase and *rosR* encoding a positive transcriptional regulator are key genes involved in the biosynthesis and regulation of EPS production. Mutation in *pssA* resulted in deficiency in EPS production and *rosR* mutation substantially decreased the amount of EPS. Both mutants induced nodules but the bacteria were unable to fix nitrogen. Defective functions of *pssA* and *rosR* mutants were fully restored by wild type copies of the respective genes. Introduction of multiple *rosR* and *pssA* gene copies on the plasmid vector pBBR1MCS-2 into five *R. leguminosarum* bv. *trifolii* nodule isolates resulted in significantly increased growth rates, EPS production and the number of nodules on clover roots. Increase in fresh and dry shoot mass of clovers and nodule occupation was also statistically significant. Interestingly, additional copies of *pssA* but particularly *rosR* gene, increased strains' competitiveness in relation to the wild type parental strains nearly twofold. Overall, experimental evidence is provided that increased amount of EPS beneficially affects *R. leguminosarum* bv. *trifolii* competitiveness and symbiosis with clover.

Keywords Competitiveness · Exopolysaccharide · *pssA* gene · *rosR* gene · *Rhizobium leguminosarum* · Symbiosis efficiency

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Introduction

Biological nitrogen fixation is a natural process of significant importance in world agriculture, in which atmospheric N_2 is reduced to ammonia (NH_3) by the enzymatic complex of nitrogenase. The greatest contribution to this process originates from the symbiosis of legumes with root-nodule bacteria, collectively called rhizobia, which represents the most efficient and intensively studied model of beneficial plant-microbe interactions (Gibson et al. 2008; Herridge et al. 2008). Microsymbionts of legumes comprise a very diverse group of soil bacteria able to induce the formation of nodules on roots of the respective host plants. This process requires a coordinated exchange of signals between plants and rhizobia, in which plant flavonoids, bacterial Nod factors and both the bacterial and plant

cell surface components all participate (Perret et al. 2000; Skorupska et al. 2006; Gibson et al. 2008). A successful early communication between these partners leads to the invasion of plant host roots, where rhizobia grow and penetrate plant tissue within the tubular infection threads. Inside the nodule cells, the bacteria are released from infection threads and differentiate into bacteroids that reduce dinitrogen to ammonia (Perret et al. 2000; Gage 2004).

Whereas the cascade of events in the nodulation process has been extensively studied and well characterized (see reviews by Perret et al. 2000; Oldroyd and Downie 2008), processes underlying nodulation competitiveness of rhizobial strains in the rhizosphere are still poorly understood. The major barrier to increasing the benefit derived from symbiotic nitrogen fixation in agriculture is the outcompetition of symbiotically effective strains by the indigenous rhizobial population that can be less efficient in nitrogen fixation. The competitiveness of rhizobia depends on several factors, among them their survival in the soil and the rhizosphere, interactions with other bacteria present in the environment and the ability to utilize different nutrients (Triplett 1990a, b; Bittinger et al. 1997; Laguerre et al. 2003; Daniels et al. 2006; Wielbo et al. 2007). All of the above influence the effectiveness of nodulation and nitrogen fixation (Toro 1996; Vlassak and Vanderleyden 1997; Djordjevic et al. 2003).

Genetic engineering of rhizobia is the most promising strategy for enhancing nodulation competitiveness. One of the best-characterized approaches in rhizobia is the introduction of genes involved in the production of antirhizobial peptide, trifolitoxin, into *Rhizobium etli* and *Sinorhizobium meliloti* strains that are unable to synthesize this peptide, which significantly increased their nodulation competitiveness and enhanced nodule occupancy (Robleto et al. 1997, 1998). Also, introduction of additional copies of *putA*, encoding proline dehydrogenase, into *S. meliloti* resulted in significant increase in competitiveness (van Dillewijn et al. 2001).

Rhizobial cell surface components play an important role in determining the symbiotic competence of rhizobia. Exopolysaccharides (EPS) and lipopolysaccharides (LPS) are essential for successful root tissue invasion and induction of effective, nitrogen-fixing nodules on host plants that form an indeterminate type of nodules (with persistent meristem) such as clover, vetch, pea or alfalfa (Becker and Pühler 1998; Fraysse et al. 2003; Mathis et al. 2005; Skorupska et al. 2006). EPS-deficient mutants or those altered in LPS structure are impaired in nodule cell invasion and nitrogen fixation (Skorupska et al. 1995; Bittinger et al. 1997; Brewin 1998; Cooper 2007). Acidic EPSs secreted in large amounts by rhizobia are species-specific heteropolymers consisting of common sugars substituted with non-carbohydrate residues (Becker and Pühler 1998; Skorupska et al. 2006; Jones et al. 2007). In the case of *R. leguminosarum*, a repeating unit is composed of five glucose, one galactose and two glucuronic acid residues, decorated by acetyl and pyruvyl groups. In *R. leguminosarum*, the key role in EPS synthesis is played by the *pssA* gene encoding the glucose-IP-transferase which initiates repeating unit synthesis. Mutation in *pssA* causes a complete inhibition of EPS production and significant disturbances in LPS synthesis in three biovars of *R. leguminosarum*, but symbiotic phenotype of the mutants depends on biovar. *R. leguminosarum* bvs. *trifolii* and *viciae pssA* mutants induce empty nodules on the respective host plants that are inefficient in nitrogen fixation (Ivashina et al. 1994; van Workum et al. 1997; Janczarek et al. 2001). In contrast, *R. leguminosarum* bv. *phaseoli pssA* mutant induces nitrogen-fixing nodules on *Phaseolus* plants, that form determinate nodules (Borthakur et al. 1986; Latchford et al. 1991). The function of *pssA* in symbiosis seems to be more general as has been partially demonstrated by comparative proteome analyses of *R. leguminosarum* wild type strain and *pssA* mutant, with the expression of several genes shown to be altered in the mutant (Guerreiro et al. 2000). Another important gene in EPS synthesis and regulation in *R. leguminosarum* bvs. *trifolii* and *viciae* is *rosR*, encoding a regulatory protein which positively regulates EPS production. *rosR* mutant produces three times less EPS than wild type strain and induces nodules incapable of nitrogen-fixation (Janczarek and Skorupska 2007). *rosR* mutant of *R. etli* formed colonies with altered morphology but retained the ability to induce nitrogen-fixing nodules on common bean (*Phaseolus vulgaris*), which forms a determinate type of nodules (Bittinger et al. 1997). However, nodulation competitiveness of this mutant was greatly reduced and, for this reason, *rosR* was suggested as a determinant of *R. etli* competitiveness (Bittinger et al. 1997).

The main objective of the current study was to assess whether multiple copies of key genes of exopolysaccharide synthesis, such as *pssA* and *rosR*, positively influence the competitiveness and symbiotic properties of rhizobia when introduced into wild type strains. We hypothesized that, by increasing the EPS production, these genes might facilitate infection of clover roots.

Materials and methods

Strains, plasmids, media and growth conditions

Bacterial strains, plasmids and oligonucleotide primers used are listed in Table 1. *R. leguminosarum* bv. *trifolii* isolates originated from nodules of *Trifolium pratense* growing in region of Lublin, Poland. Nodules were surface sterilized with 0.1% LiCl, washed with water, sterilized with 70% ethanol, again washed with water, crushed in 0.9% NaCl solution and grown on 79CA medium at 28°C (Vincent 1970). Strains isolated from nodules were purified by successive isolation of single colonies and assayed for symbiotic activity. For growth assays, the rhizobia were cultured for 60 h at 28°C in M1 minimal medium (Sambrook et al. 1989), supplemented with kanamycin when needed. Every 12 h, the optical density (OD_{600}) of cultures was measured. *Escherichia coli* strains were routinely grown on Luria-Bertani (LB) medium at 37°C (Sambrook et al. 1989). Antibiotics for *E. coli* and *R. leguminosarum* cultures were used at the following final concentrations: kanamycin, 40 μg ml^{-1}; rifampicin 40 μg ml^{-1}; ampicillin, 100 μg ml^{-1}; tetracycline 10 μg ml^{-1}; nalidixic acid, 40 μg ml^{-1}.

DNA methods and sequence analysis

Standard techniques were used for plasmid and genomic DNA isolation, restriction enzyme digestion, cloning and transformation (Sambrook et al. 1989). Separations of large rhizobial plasmids were performed according to the Eckhardt protocol (1978). For PCR amplifications, Ready *Taq* PCR Reaction Mix (Sigma) and plasmid or genomic DNA isolated from *E. coli* and *R. leguminosarum* bv. *trifolii* strains were used as templates. Primers FGPS1490 (Navarro et al. 1992) and FGPL132′ (Ponsonnet and Nesme 1994) were used to amplify the 16S–23S rRNA intergenic transcribed sequence (ITS) of *R. leguminosarum* bv. *trifolii* isolates according to Laguerre et al. (1996). In PCR-RFLP analysis, PCR products were digested with *Taq*I enzyme (FERMENTAS) at 65°C and the restriction fragments were separated by 3% agarose gel electrophoresis. Amplicons used for *rosR* and *pssA* mutagenesis and the plasmid constructs were sequenced using the BigDye terminator cycle sequencing kit (Applied Biosystems) and the ABI Prism 310 sequencer. Database searches were done with the BLAST and FASTA programs available from the National Center for Biotechnology Information (Bethesda, MD, USA) and European Bioinformatic Institute (Hinxton, UK).

Mutagenesis of *pssA* and *rosR* genes

For mutagenesis of *pssA* and *rosR* genes, plasmids pM34, containing the entire *pssA*, and pM31, with entire *rosR*, as targets and the EZ::TN^{TM} < KAN-2 > Insertion Kit (Epicentre Technology) which enables generation of random insertions of mini-Tn*5* transposon into target DNA were used according to manufacturer's instruction. The location of mini-Tn*5* transposon was established by restriction analyses and DNA sequencing. Of 25 Km^r derivatives of pM34, two plasmids containing mini-Tn*5* inserted in the central part of the coding region were chosen for further study: pMT37 with insertion in position 502–503 bp, and pMT58, in position 363–364 bp of *pssA* (accession no. AF316883), respectively. 4.6 kb *Eco*RI inserts of pMT37 and pMT58 were cloned into the appropriate site of pSUP202 vector, resulting in plasmids pMSUP37 and pMSUP58, respectively. These constructs were introduced into *E. coli* S17-1 by transformation and subsequently into Rt24.2 by two-parental conjugation. Transconjugants were selected on 79CA medium with kanamycin and rifampicin. A clone named Rt5819, which formed small colonies with non-mucoid morphology, was isolated. In *rosR* mutagenesis, of 16 Km^r derivatives of pM31 only one plasmid, named pMT21, carried mini-Tn*5* transposon inside the coding region, in position 151–152 bp of ORF (accession no. AY683453). Using pMT21 carrying the *rosR*-mini-Tn*5* insertion as a template with pEP7 and RR1 primers containing engineered *Eco*RI and *Xba*I recognition sites, 1.5 kb fragment was PCR

Table 1 Bacterial strains and plasmids used in this study

Strain, plasmid or oligonucleotide primers	Relevant characteristics	Source or reference
R. leguminosarum		
bv. trifolii 24.2	Wild type, Rif^r, Nx^r	Janczarek and Skorupska (2007)
MJ12, MJ21, MJ43, MJ51	Clover nodules isolates	Janczarek et al. (2009)
Rt5819	Rt24.2 derivative carrying mini-Tn5 in 363–364 bp position of *pssA*	This work
Rt2472	Rt24.2 derivative carrying mini-Tn5 in 151–152 bp position of *rosR*	This work
E. coli		
DH5α	*supE*44 Δ*lac*U169 (φ80 *lacZ*Δ M15) *hsdR*17 *recA*1*endA*1*gyrA*96 *thi*-1 *relA*1	Sambrook et al. (1989)
S17-1	294 derivative RP4-2Tc::Mu-Km::Tn7 chromosomally integrated	Simon et al. (1983)
Plasmids		
pBBR1MCS-2 (in this work named as pBK1)	*mob*, *lacZα*, Km^r	Kovach et al. (1995)
pBBR1MCS-5	*mob*, *lacZα*, Gm^r	Kovach et al. (1995)
pSUP202	pBR325 derivative, *mob*, Cm^r Tc^r Ap^r	Simon et al. (1983)
pRK7813	IncP, *mob*, cosmid vector, Tc^r	Stanley et al. (1987)
pK19mobGII	*mob*, *lacZα*, *gusA*, Km^r	Katzen et al. (1999)
pJBA21Tc	pJB321 derivative carrying constitutively expressed *gusA*, Tc^r	Wielbo et al. (2007)
pM31	pUC19 with 1,101-bp *Eco*RI fragment containing *rosR* of Rt24.2	Janczarek and Skorupska (2007)
pM34	pUC19 with 3,400-bp *Eco*RI fragment containing *pssA* of Rt24.2	This work
pMSUP37	pSUP202 containing 4,621-bp *Eco*RI fragment with mini-Tn5 inserted in position 502–503 bp of *pssA* ORF	This work
pMSUP58	pSUP202 containing 4,621-bp *Eco*RI fragment with mini-Tn5 inserted in position 502–503 bp of *pssA* ORF	This work
pMK21	pK19mobGII containing 1,504-bp *Eco*RI-*Xba*I fragment with mini-Tn5 inserted in position 151–152 bp of *rosR*	This work
pBR1	pBBR1MCS-2 with 1,100-bp *Eco*RI- *Bam*HI fragment containing *rosR*	This work
pBA1	pBBR1MCS-2 with 1,450-bp *Eco*RI- *Hind*III fragment containing *pssA*	This work
pM6	pRK7813 with 3,400-bp *Eco*RI fragment containing *pssA* of Rt24.2	This work
pM16	pBBR1MCS-5 with 3,400-bp *Eco*RI fragment containing *pssA* of Rt24.2	This work
pRC24	pRK7813 with 1,174-bp *BamHI* fragment containing *rosR* of Rt24.2	Janczarek and Skorupska (2007)
pBR24	pBBR1MCS-5 with 1,174-bp *BamHI* fragment containing *rosR* of Rt24.2	Janczarek and Skorupska (2007)
Oligonucleotide primers	Sequence (5′-3′)[a]	
FGPS1490	TGCGGCTGGATCACCTCCTT	Navarro et al. (1992)
FGPL132′	CCGGGTTTCCCCATTCGG	Ponsonnet and Nesme (1994)

Table 1 continued

Strain, plasmid or oligonucleotide primers	Relevant characteristics	Source or reference
pssAIN1	AGATCGTTGAATTCCGGCAAGCG	This work
pssAD1	ACGGATCCACCGGTGCCGGCTGC	This work
pEP2	GCCCCTGAATTCTTCATCTGTCA	Janczarek and Skorupska (2007)
pEP7	GCGTGGAATTCAAAGGCCGTTTG	
RR1	CGCATTCTAGACATGTGATCTGC	
RosD	TCGGATCCTGTCGGCAAAGCATA	
rosRbF	GCGATCGTCGAAAAGCAGAAG	This work
rosRbR	GAGAGGCTGTGATGCGTCATC	This work
pssAaF	CACGGCTTCGACAGTGATCG	This work
pssAaR	TTTGGCAGGGCTTCACAGG	This work
dnaKbF	CGAAGGTTATCGAGAATGCGG	This work
dnaKbR	CAAAGAGCGTGTTCGTCGG	This work

[a] Sequences for *Eco*RI, *Bam*HI and *Xba*I restriction sites are underlined

amplified. This amplicon was inserted into the respective sites of pK19mobGII suicide vector, resulting in pMK21. The construct was transferred from *E. coli* S-17 to Rt24.2 by conjugation and the anticipated transconjugants were selected on 79CA medium with kanamycin and rifampicin. The clone named Rt2472, that showed altered, wrinkled colony morphology, was isolated. The correctness of homologous recombination inside the *pssA* and *rosR* genes was verified by PCR analysis and Southern hybridization.

Cloning of *rosR* and *pssA* genes into pBBR1MCS-2 plasmid

To construct broad-host-range plasmids bearing *rosR* and *pssA*, the medium-copy plasmid pBBR1MCS-2 was used. *rosR* gene was PCR amplified using pM31 plasmid as a template, forward primer pEP2 with *Eco*RI recognition site and reverse primer RosD with *Bam*HI site. This amplicon was cloned into the corresponding sites in pBBR1MCS-2, yielding pBR1 plasmid. *pssA* gene was PCR amplified using pM34 plasmid as a template, forward primer pssAIN1 with *Eco*RI site and reverse primer pssAD1. The PCR product, which possessed *Hin*dIII internal site, was digested with *Eco*RI and *Hin*dIII and cloned into corresponding sites of pBBR1MCS-2, resulting in pBA1. The inserts of both pBA1 and pBR1 plasmids contained ORFs with their own promoters. The constructs were verified by sequencing and then plasmids pBR1, pBA1 and pBBR1MCS-2 (in this work: pBK1) were introduced into Rt24.2 and *R. leguminosarum* bv. *trifolii* field isolates by electroporation (Garg et al. 1999). The stability of these plasmids in rhizobia was established to be on average 95% in the absence of antibiotic.

Determination of pBR1 and pBA1 copy numbers by real-time PCR

Real-time PCR products were amplified on Applied Biosystems ABI PRISM 7500 Real Time PCR System using SYBR green. PCR was performed in 25-µl reaction mixtures containing the following components: 1 × concentrated commercial *Taq* polymerase buffer supplied with the enzyme (Invitrogen), 4 mM $MgCl_2$, 0.01% Tween 20, 0.8% glycerol, 1:30000 diluted SYBR Green I® (Sigma), 5% DMSO, 0.5 ng/µl acetylated BSA (Sigma), dNTP 400 nM each, 0.625 U *Taq* polymerase (Invitrogen), 200 × diluted a reference dye ROX (Invitrogen), rosRbF and rosRbR primers for *rosR* gene, pssAaF and pssAaR for *pssA*, dnaKbF and dnaKbR for *dnaK* at optimized concentrations (Table 1) and 0.9–24 ng of a total genomic DNA prepared from Rt24.2, Rt24.2(pBR1) and Rt24.2(pBA1) strains (Sambrook et al. 1989). Amplification conditions for all three

primer sets were as follows: an initial denaturation at 95°C for 3 min and then 40 cycles of denaturation (95°C for 15 s) and annealing/extension (60°C for 1 min) with fluorescence reading. The values are means of two independent experiments.

Plant tests

Red clover (*Trifolium pratense* cv. Diana) seeds were surface sterilized, germinated and grown on Fåhraeus medium (Vincent 1970) slants. 5-day-old plants were inoculated by bacterial suspensions of OD_{600} 0.1 (approx. 10^7 cells/ml; 200 µl/plant). Plants were grown in a greenhouse under natural light supplemented with artificial light (14 h day at 24°C and 10 h night at 18°C). The clover plants were inspected for root nodule formation and harvested after 4 weeks. Wet and dry masses of clover shoots were estimated by weighing. The results of plant tests were subjected to analysis of variance (a linear ANOVA model) with three replicates, using Microsoft® Excel 2000 for Windows. P value ≤ 0.05 was considered significant (Armitage and Berry 1987).

To study a nodule occupancy, plasmid pJBA21Tc containing a constitutively expressed *gusA* (Wielbo et al. 2007) was introduced into *R. leguminosarum* bv. *trifolii* strain 24.2 by electroporation and used for clover inoculation. Nodules were surface sterilized, crushed and cultured on 79CA medium supplemented with tetracycline. Single colonies were counted.

Plant competition assay

Red clover plants were grown on slants with Fahraeus medium (1 seedling/tube). Five-day-old seedlings were inoculated with mixtures of wild type strains and their derivatives harboring pBK1, pBR1 or pBA1 plasmids (1:1 v/v). In total, 200 µl mixed bacterial suspension, OD_{600} 0.1 ($\sim 0.2 \times 10^7$ CFU/ml), was added to each slant. Thirty slants per treatment were used. Three weeks after inoculation, nodules were surface sterilized and crushed in 50 µl sterile water, and 15 µl aliquots of bacterial suspensions were plated on 79CA medium both with and without kanamycin. Colonies growing on the respective media were counted, and the data are presented as a percent of nodules occupied by a modified strain (colonies Km^r) versus a wild type strain (colonies Km^s). In the second experiment, seedlings were inoculated with mixtures of two strains (1:1 v/v), which contained Rt24.2(pJBA21Tc) with constitutively expressed *gusA* (control) and one of the tested wild type strains or their derivatives harboring pBK1, pBR1 or pBA1 plasmids. After 3 weeks, plant roots of 40 plants for each treatment were harvested and stained for β-glucuronidase activity (Wilson et al. 1995). The number of white nodules colonized by non-tagged strains and the number of blue nodules colonized by tagged Rt24.2(pJBA21Tc) strain were estimated.

To study coinoculation of a single nodule by two strains, bacteria were isolated from ten independent nodules per treatment. Bacterial suspensions were diluted and plated on 79CA medium. Then, single colonies were streaked on the same medium both with and without kanamycin (the first experiment) or on a medium both with and without tetracycline (the second experiment).

EPS isolation

For EPS isolation, 10-ml cultures of rhizobia were grown in 79CA medium with 0.5% glycerol for 2 days at 28°C in a rotary shaker. EPS was precipitated from culture supernatants with 10 vol. of 96% ethanol and after re-dissolving in water the precipitates were analyzed as described earlier (Janczarek and Skorupska 2007). Total sugar content was calculated as glucose equivalents.

Results

Characteristics of *R. leguminosarum* bv. *trifolii* *pssA* and *rosR* mutants

A mutation in the central coding region of *pssA* was introduced by random mini-Tn*5* mutagenesis and the Rt5819 mutant that formed nonmucoid colonies on agar medium was isolated. By the same method, the Rt2472 *rosR*-miniTn*5* mutant forming altered, wrinkled colonies was isolated. Both mutants were characterized with respect to exopolysaccharide synthesis and symbiotic properties (Table 2). Rt5819 *pssA* mutant was totally deficient in EPS production and induced low quantity of non infected, empty or almost empty nodules inefficient in nitrogen fixation.

Table 2 Symbiotic properties of *R. leguminosarum* bv. *trifolii* 24.2 *rosR* and *pssA* mutants

Strains	Nodule no. per plant[a]	Fresh shoot mass (mg plant^{-1})[a]	Dry shoot mass (mg plant^{-1})[a]	Nodule occupancy $(\times 10^4)$[b]	EPS (μg Glc μg protein^{-1})[c] (%)
Rt24.2 (wild type)	9.1 ± 2.5	53.9 ± 11.1	5.95 ± 0.3	35.2 ± 12	0.96 ± 0.03 (100%)
Rt5819 (*pssA*$^-$)	3.2 ± 2.2	32.1 ± 6.1	3.75 ± 0.2	0.2 ± 0.1	0 (0%)
Rt5819 (pM6)	10.6 ± 3.8	53.1 ± 6.8	6.05 ± 0.3	32.0 ± 9.2	1.25 ± 0.03 (129%)
Rt5819 (pM16)	12.2 ± 4.2	55.2 ± 12.2	6.10 ± 0.4	38.5 ± 12	1.76 ± 0.04 (183%)
Rt2472 (*rosR*$^-$)	4.1 ± 1.9	36.1 ± 11.2	4.10 ± 0.2	3.1 ± 0.9	0.32 ± 0.02 (33.3%)
Rt2472 (pRC24)	10.7 ± 3.3	51.9 ± 10.8	5.9 ± 0.3	36.0 ± 5.2	1.47 ± 0.04 (153%)
Rt2472 (pBR24)	18.1 ± 5.8	53.6 ± 9.8	6.1 ± 0.4	38.8 ± 15	1.90 ± 0.05 (198%)

[a] Values are mean of 50 plants ± standard deviation

[b] Values are mean of 6 nodules

[c] Values are the means ± standard errors of the mean for triplicate independent assays

rosR mutant produced three times less EPS, elicited about two times less nodules, which were occupied by ten times fewer bacteria, in relation to Rt24.2 wild type strain. A substantial decrease of fresh and dry shoot mass of plants infected by Rt2472 was noticed in comparison to the wild type strain.

For complementation analysis, wild type copies of *pssA* and *rosR* genes cloned into low copy vector pRK7813 and medium copy pBBR1MCS-5 plasmid were introduced into Rt5819 *pssA* and Rt2472 *rosR* mutants. In both mutants, the allelic wild type genes not only restored EPS production but also essentially increased the level of EPS synthesis in comparison to Rt24.2 wild type strain (Table 2). Particularly, about twofold increase in EPS production was observed in the case of *pssA* and *rosR* mutants harboring additional copies of the respective genes on pBBR1MCS-5. Also, an essential increase in nodule number was induced on clover roots by Rt5819 and Rt2472 mutants harboring plasmids bearing *pssA* or *rosR* genes, respectively, and two times more nodules than in wild type strain Rt24.2 were observed in the case of clover inoculation by Rt2472(pBR24). However, both plant fresh and dry masses and nodule occupation were comparable to wild type strain Rt24.2 (Table 2). These results confirmed earlier observations that EPS production is indispensable for infection and for effective symbiosis of *R. leguminosarum* bv. *trifolii* with clover and demonstrated that increased production of this polysaccharide directed by multiple copies of *rosR* and *pssA* genes resulted in enhancement of nodulation.

Effect of multiple copies of *rosR* and *pssA* on growth, EPS production and symbiosis of *R. leguminosarum* bv. *trifolii* field isolates

Because our complementation experiments indicated that multiple copies of *pssA* and *rosR* genes caused enhancement of EPS production and clover nodulation, we assessed the effect of these genes on Rt24.2 laboratory strain and four clover nodule isolates. The strains MJ12, MJ21, MJ43 and MJ51 were chosen from the collection of *R. leguminosarum* bv. *trifolii* nodule isolates. They moderately varied in symbiotic efficiency, as was shown by comparing wet and dry shoot mass of clover plants, nodule number and occupation (Table 3). Particularly, strain MJ12 stood out because of lower infectivity (90% plants were nodulated after 2 weeks) and lower shoot mass when compared to the other studied strains. These strains significantly differed in plasmid patterns, having 3–5 plasmids (Fig. 1a). PCR-RFLP polymorphism studies of 16S–23S ITS employing *Taq*I restriction enzyme demonstrated significant chromosomal diversity and four distinct patterns were observed among these strains. The PCR-RFLP patterns in MJ12 and MJ21 isolates were identical (Fig. 1b).

The plasmids pBA1 and pBR1, carrying *pssA* and *rosR* genes with their own promoters, respectively, and derived from broad host range vector pBBR1MCS-2 were introduced into Rt24.2 laboratory strain and MJ12, MJ21, MJ43, MJ51 isolates. Plasmid pBBR1MCS-2 (here named pBK1) was also introduced into all strains and was treated as a

Table 3 Symbiotic properties of *R. leguminosarum* bv. *trifolii* strains carrying multiple copies of *rosR* and *pssA* genes

Strains and plasmids	Nodule no. per plant[a]	Fresh shoot mass (mg plant^{-1})[a]	Dry shoot mass (mg plant^{-1})[a]	Nodule occupancy ($\times 10^4$)[b]
Rt24.2	$9.6 \pm 2.5_{B}$	$54.6 \pm 7.1_{AC}$	$6.0 \pm 0.3_{AC}$	$34.3 \pm 4.3_{C}$
Rt24.2 (pBK1)	$8.9 \pm 2.1_{B}$	$51.6 \pm 5.6_{BC}$	$5.9 \pm 0.2_{BC}$	$4.2 \pm 0.3_{D}$
Rt24.2 (pBA1)	$13.0 \pm 5.6_{A}$	$57.3 \pm 6.7_{A}$	$6.5 \pm 0.4_{AC}$	$34.9 \pm 2.5_{BC}$
Rt24.2 (pBR1)	$14.2 \pm 4.7_{A}$	$58.9 \pm 5.5_{A}$	$6.6 \pm 0.3_{A}$	$39.6 \pm 1.7_{A}$
MJ12	$6.8 \pm 2.0_{B}$	$44.9 \pm 5.1_{B}$	$4.9 \pm 0.2_{B}$	$41.4 \pm 9.7_{B}$
MJ12 (pBK1)	$6.9 \pm 2.3_{B}$	$43.6 \pm 6.0_{B}$	$4.7 \pm 0.2_{B}$	$5.0 \pm 2.3_{C}$
MJ12 (pBA1)	$10.7 \pm 3.1_{A}$	$51.3 \pm 5.0_{A}$	$5.6 \pm 0.3_{A}$	$62.7 \pm 25.4_{AB}$
MJ12 (pBR1)	$11.6 \pm 3.4_{A}$	$52.7 \pm 4.4_{A}$	$5.6 \pm 0.2_{A}$	$68.9 \pm 11.1_{A}$
MJ21	$9.4 \pm 2.6_{BC}$	$60.8 \pm 8.8_{AC}$	$6.4 \pm 0.3_{B}$	$35.3 \pm 8.2_{AD}$
MJ21(pBK1)	$8.2 \pm 2.4_{B}$	$57.1 \pm 6.3_{BC}$	$6.1 \pm 0.3_{B}$	$5.1 \pm 1.4_{C}$
MJ21(pBA1)	$11.1 \pm 3.1_{AC}$	$64.5 \pm 7.4_{A}$	$6.9 \pm 0.2_{A}$	$29.9 \pm 9.4_{BD}$
MJ21(pBR1)	$12.0 \pm 2.7_{A}$	$66.3 \pm 6.0_{A}$	$7.1 \pm 0.2_{A}$	$42.3 \pm 6.5_{A}$
MJ43	$9.1 \pm 2.6_{B}$	$56.6 \pm 11.7_{AC}$	$6.2 \pm 0.3_{BC}$	$35.5 \pm 4.7_{A}$
MJ43 (pBK1)	$8.5 \pm 2.5_{B}$	$55.3 \pm 6.5_{BC}$	$6.0 \pm 0.2_{B}$	$3.7 \pm 0.6_{B}$
MJ43 (pBA1)	$11.8 \pm 2.7_{A}$	$63.5 \pm 8.1_{A}$	$6.9 \pm 0.3_{A}$	$34.2 \pm 6.0_{A}$
MJ43 (pBR1)	$12.7 \pm 3.0_{A}$	$62.9 \pm 8.2_{A}$	$6.6 \pm 0.3_{AC}$	$39.7 \pm 9.0_{A}$
MJ51	$9.6 \pm 3.5_{BC}$	$61.1 \pm 9.4_{AC}$	$6.5 \pm 0.2_{B}$	$51.8 \pm 24.2_{A}$
MJ51(pBK1)	$8.8 \pm 3.0_{B}$	$59.7 \pm 7.2_{BC}$	$6.3 \pm 0.3_{B}$	$5.2 \pm 0.4_{B}$
MJ51(pBA1)	$14.4 \pm 6.7_{A}$	$63.9 \pm 6.4_{AC}$	$7.1 \pm 0.2_{A}$	$48.4 \pm 5.6_{A}$
MJ51(pBR1)	$12.4 \pm 4.3_{AC}$	$64.8 \pm 4.9_{A}$	$6.9 \pm 0.2_{A}$	$52.8 \pm 17.3_{A}$

Means within the column (for particular strains) followed by different capital letters have significantly different values ($P \leq 0.05$)

[a] 28 days after infection. Values are mean ± standard deviation of 50 plants

[b] Values are mean ± standard deviation of 6 nodules

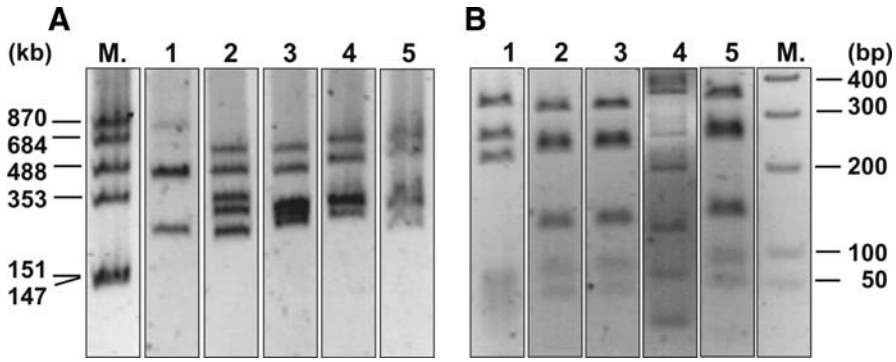

Fig. 1 Plasmid profiles of *Rhizobium leguminosarum* bv. *trifolii* isolates obtained by Eckhardt's method (**a**). M—plasmids of *Rhizobium leguminosarum* bv. *viciae* strain 3,841 used as a marker of plasmid sizes. *Rhizobium leguminosarum* bv. *trifolii* isolates: (1) Rt24.2, (2) MJ12, (3) MJ21, (4) MJ43, (5) MJ51. **b** PCR-RFLP of 16S-23S rDNA ITS region. PCR products were digested with *Taq*I restrictase. Isolates: (1) Rt24.2, (2) MJ12, (3) MJ21, (4) MJ43, (5) MJ51. M—molecular mass standard

control. The copy numbers of pBA1 and pBR1 in Rt24.2 strain were estimated using Real-time PCR and primers complementary to *rosR*, *pssA* and *dnaK* as an internal control. All three genes were present in single copies in the genome of Rt24.2, which was confirmed by the *pssA/dnaK* ratio 1.02 ± 0.13 and

the *rosR/dnaK* ratio 0.97 ± 0.14, respectively. In Rt24.2 carrying pBA1, the *pssA/dnaK* ratio was 6.96 ± 1.05 (*rosR/dnaK* ratio 0.96 ± 0.12) indicating that the plasmid with *pssA* was present in six copies. Rt24.2(pBR1) strain showed the *rosR/dnaK* ratio of 8.42 ± 1.12 (*pssA/dnaK* ratio 1.33 ± 0.19) confirming the presence of above seven copies of pBR1 in Rt24.2 background.

The effect of additional *pssA* and *rosR* gene copies on bacterial growth in complete 79CA and minimal M1 media was examined (Fig. 2). Growth rates of all strains in 79CA medium were similar and reached OD_{600} ~0.7–0.8 after 24 h (data not shown). On the other hand, the growth rates of these strains in M1 medium differed markedly (Fig. 2a). Strains MJ21 and Rt24.2 were identified as auxotrophic and a mixture of vitamins was added to M1 growth medium in further experiments (Fig. 2b, d). The presence of the control plasmid pBK1 was accompanied by a slightly decreased growth of the strains in comparison to wild types, probably because of the antibiotic present in the growth medium. In all rhizobial strains grown in M1 medium, introduction of additional copies of *pssA* and *rosR* genes resulted in a substantial increase of growth rates and this effect was most visible in the case of rhizobia bearing plasmid pBR1 carrying *rosR* (Fig. 2b–f).

Next, the EPS production was examined in *R. leguminosarum* bv. *trifolii* wild type strains and their derivatives carrying additional copies of *pssA* and *rosR* genes (Fig. 3). Wild type strains showed moderate diversity in the amount of EPS production, ranging from 0.75 μg glucose μg protein^{-1} in MJ21 to 1.02 μg glucose μg protein^{-1} in MJ51. Additional copies of *pssA* on plasmid pBA1 lead to increased EPS production in wild type derivatives an average 1.65 times. The additional copies of *rosR* (plasmid pBR1) resulted in increased EPS production in the range from 1.86-fold in MJ51 and Rt24.2 to 2.03-fold in MJ21. These results indicated that multiple copies of both *rosR* and *pssA* genes were associated with a substantially increased EPS synthesis, however, the effect of multiple *rosR* copies was noticeably greater than *pssA* in all strains studied.

Symbiotic properties of *R. leguminosarum* bv. *trifolii* strains carrying additional *rosR* and *pssA* gene copies are shown in Table 3. The presence of control plasmid pBK1 in the analyzed strains did not substantially affect nodule number and fresh or dry shoot mass of plants (Table 3). All tested strains carrying additional copies of *pssA* and *rosR* induced higher number of nodules on clover roots in comparison to strains with the control plasmid, and the highest increase was observed in the case of MJ12 strain (155% for *pssA* and 168% for *rosR*, respectively) (statistically significant data, P values ≤ 0.05). The additional copies of *pssA* and *rosR* significantly influenced wet and dry shoot mass of plants, increasing the weight of plant shoots up to 118.5% in MJ12 with *pssA*, and 121.5% in MJ12 with *rosR*. Generally, rhizobia with multiple copies of *rosR* displayed an increase in nodulation and symbiotic efficiency to a greater extent than strains harboring *pssA* gene. Multiple copies of *pssA* and *rosR* most effectively improved symbiotic properties of strain MJ12, which initially showed the lowest level of nodulation and symbiotic efficiency among all the tested strains (Table 3).

For unknown reasons, the presence of plasmid pBK1 strongly affected nodule occupancy, decreasing the number of bacteria in the nodules 7–10 times (Table 3). In general, multiple copies of *pssA* influenced the nodule occupancy, increasing the number of bacteria in the nodules 6–12 times depending on the isolate, in comparison to strains infected by rhizobia harboring the control plasmid. Strains with additional copies of *rosR* demonstrated increased nodule occupancy (8.5–11.8 times), indicating that this gene significantly influenced nodule occupancy. When the quantity of rhizobia inside nodules colonized by strains containing multiple *rosR* and *pssA* copies was compared to strains without plasmid pBK1, only in the case of MJ12 strain was the bacterial number significantly increased.

Effect of additional copies of *pssA* and *rosR* on competitiveness

To study the effect of *pssA* and *rosR* gene copies on competitiveness of *R. leguminosarum* bv. *trifolii*, the cultures of wild type strains were mixed with their derivatives harboring pBK1, pBA1 or pBR1 plasmids in 1:1 ratio and the mixtures were used to inoculate clover seedlings. Data are presented as percentages of Km^r and Km^s colonies obtained from 60 nodules for each treatment (Fig. 4a).

It was established that rhizobial strains with pBK1 control plasmid colonized about 40% of nodules,

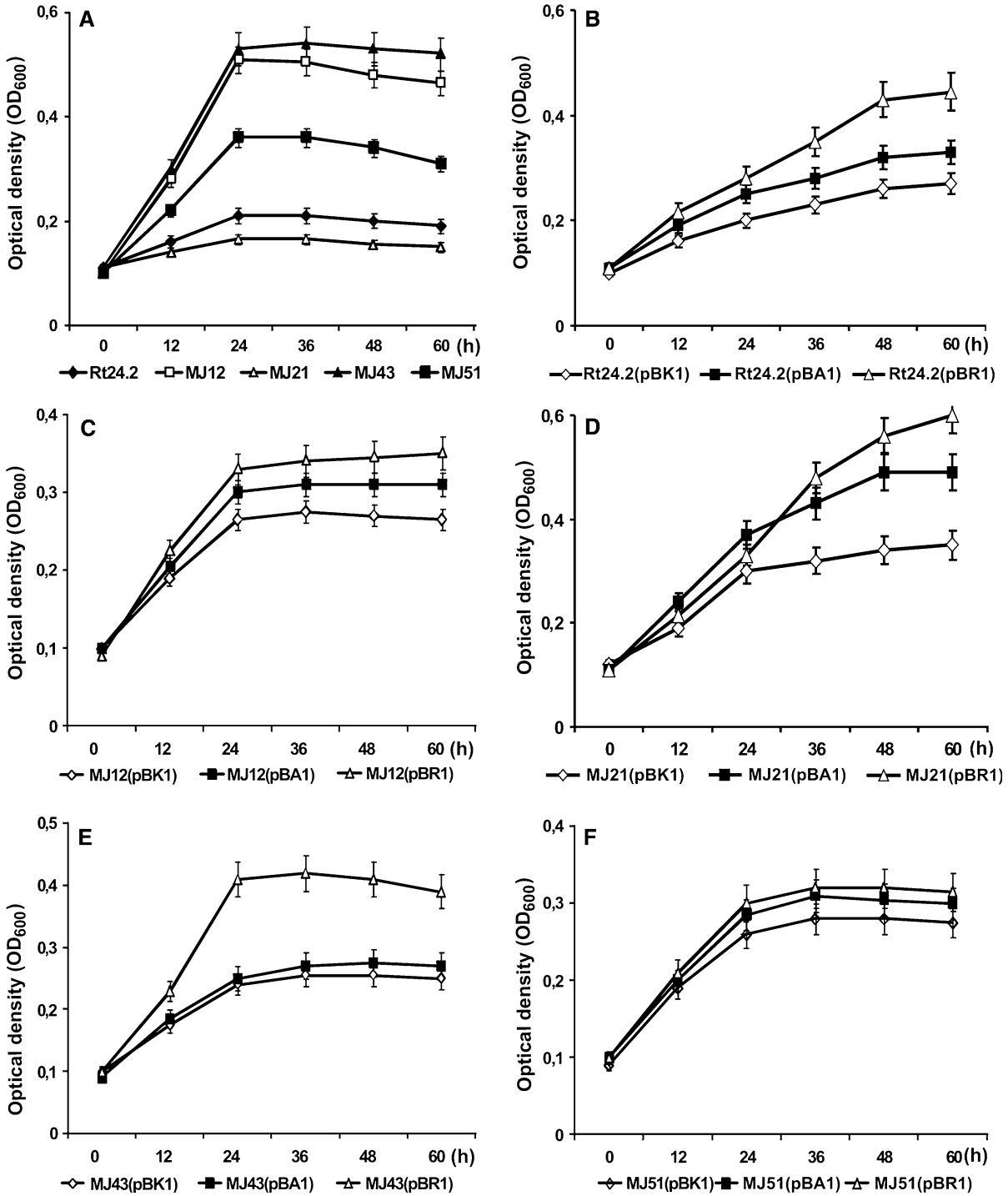

Fig. 2 Growth kinetics of *R. leguminosarum* bv. *trifolii* wild type strains and their derivatives carrying plasmids pBBR1MCS-2 (pBK1), pBA1 and pBR1 on minimal M1 medium supplemented with kanamycin (**a**–**e**). Culture media of MJ21 and Rt24.2 strains were additionally supplemented with Dilworth's vitamins (**b**, **e**). Strains carrying a control plasmid pBK1 (*open rhombus*), pBR1 (*open triangle*) and pBA1 (*filled square*). *Values* represent averages of at least three independent experiments

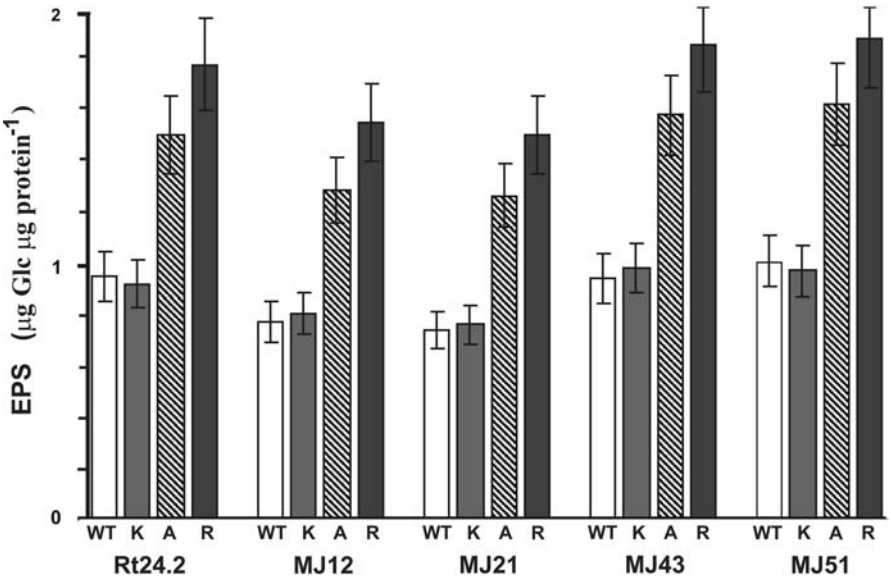

Fig. 3 Effect of multiple copies of *rosR* and *pssA* on EPS production in *Rhizobium leguminosarum* bv. *trifolii*. Wild type strains and their derivatives carrying pBR1, pBA1 and pBK1 plasmids were cultured in 79CA medium supplemented with 1% glycerol as a carbon source and kanamycin for 48 h. The strains were marked as follows: pBK1 (K), pBA1 (A), pBR1 (R). *Data* shown are the means of three replicates ±SD

with the exception of MJ12(pBK1) which occupied 56% of nodules. These results showed that to some extent (~10%) pBK1 decreased nodulation competitiveness of the majority of the studied strains. In the case of strains harboring multiple copies of *pssA* and *rosR* genes, nodule colonization was substantially increased in relation to the parental strains as demonstrated by 78–84% in the case of strains bearing pBA1, and from 85 to 91% in strains with pBR1. Thus, *pssA* and *rosR* genes positively influenced competitiveness of the studied rhizobia.

In the second approach, nodulation competitiveness of wild type strains and their derivatives carrying pBK1, pBA1 and pBR1 plasmids was examined in relation to laboratory strain Rt24.2 rendered tractable with plasmid pJBA21Tc containing *gusA* (Fig. 4b). The most competitive were MJ51 and MJ43 wild type strains, which occupied 86 and 84% of nodules, respectively. MJ12 and MJ21 colonized about 70% of nodules. The competitiveness of Rt24.2 laboratory strain was low, ranging from 14 to 29% of nodules, depending of the tested strains. In this experiment, the presence of control plasmid pBK1 essentially did not affect the strains' competitiveness. Additional copies of *pssA* and *rosR* were associated with a very high increase in nodule colonization, especially in the case of Rt24.2 (up to 94 and 96%, respectively). Additional copies of *rosR* influenced the competitiveness of all strains to a greater extent and rhizobia carrying additional copies of this gene occupied from 95 to 98% nodules. These results (Fig. 4a, b) confirmed that both *pssA* and *rosR* present in multiple copies significantly improve the competitiveness of *R. leguminosarum* bv. *trifolii*.

To verify the possibility of nodule coinfection under our experimental conditions, we performed an additional experiment in which nodule occupancy by both strains (Km^r/Km^s and Gus^+/Gus^-) in clover inoculation was estimated. Bacteria from 10 nodules from each treatment were diluted and plated on 79CA medium without antibiotic to obtain single colonies. Then, 50 colonies derived from each nodule were streaked on the medium with and without antibiotic. We observed that the majority of the nodules were occupied exclusively by a single strain (75% on average). In the remaining nodules, both strains were detected, but the bacterial number of the second strain was very low (in a range from 2 to 6%) and did not exceed 6% in an individual nodule. These results confirmed that under sterile laboratory conditions, mixed occupancy of nodules was a relatively rare phenomenon, which did not have a significant influence on total nodulation competitiveness results.

Discussion

Exopolysaccharide (EPS) is a species-specific heteropolymer required for root hair curling, proper infection thread formation, bacteria release, bacteroid

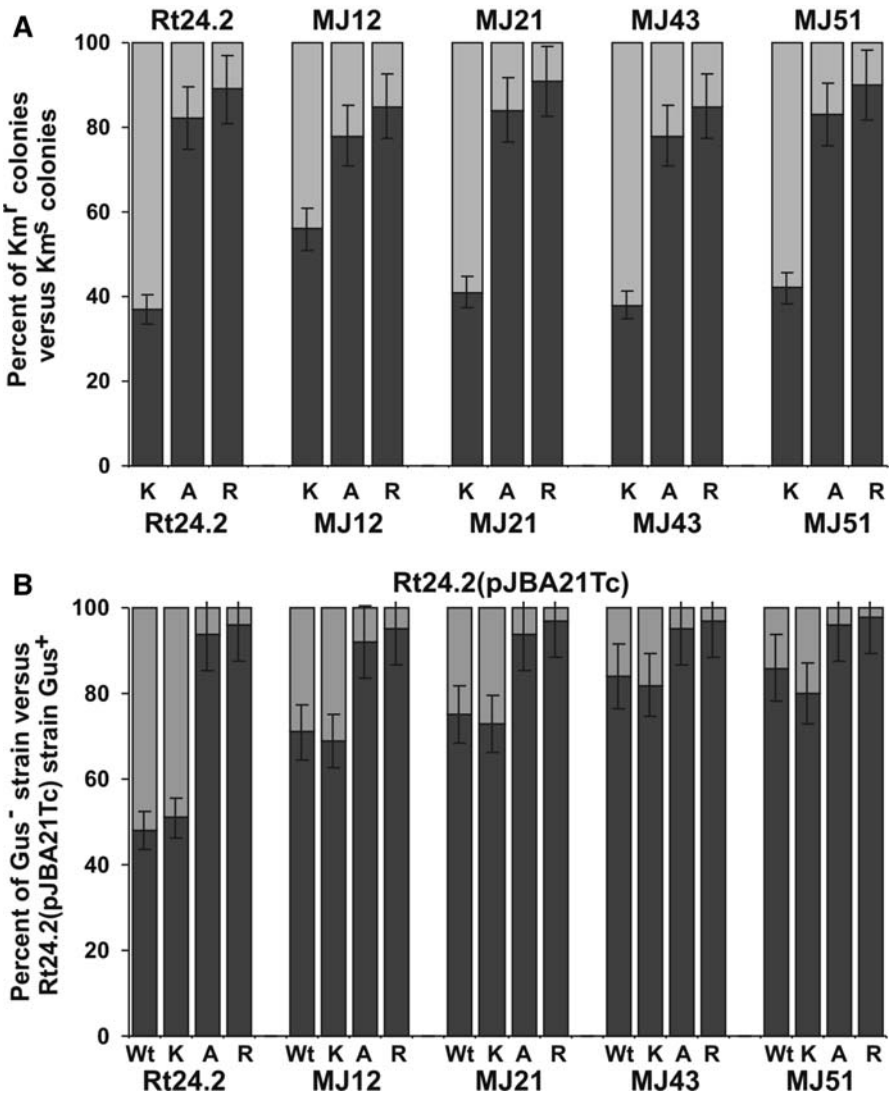

Fig. 4 Effect of multiple copies of *rosR* and *pssA* on competitiveness of *Rhizobium leguminosarum* bv. *trifolii*. Percentage of nodules colonized by particular strains 21 d after infection is shown. **a** The *lower*, *dark* segment of each *bar* represents the percent of nodules occupied by strains bearing control plasmid pBK1 (K), pBA1 (A) and pBR1 (R) plasmids. The *upper*, *light* segment of each *bar* represents the percent of nodules colonized by *R. leguminosarum* bv. *trifolii* wild type strains. **b** The *lower*, *dark* segment of each *bar* represents the percent of nodules occupied by wild type strains (WT) and strains bearing control plasmid pBK1 (K), pBA1 (A) and pBR1 (R). The *upper*, *light* segment of each *bar* represents the percent of nodules occupied by control strain Rt24.2(pJBA21Tc) (Gus^+). *Data* shown are the means of three replicates ±SD

development and the effective nodulation of host plants which form an indeterminate type of nodules (Rolfe et al. 1996; Becker and Pühler 1998; van Workum et al. 1998, Laus et al. 2005; Skorupska et al. 2006). Moreover, several lines of evidence indicate that EPS, among other surface polysaccharides, plays an essential role in protection against host plant defense (D'Haeze and Holsters 2004). The multiple functions of EPS in the symbiotic interaction also imply a possible importance of its quantity in adaptation to changing environmental conditions and in competitiveness of rhizobia. *R. leguminosarum* mutants affected in the *pss* genes crucial for EPS biosynthesis, such as *pssA* gene, are totally deficient in EPS production and induce non-infected, empty nodules with symptoms of plant defense reaction (Ivashina et al. 1994; van Workum et al. 1997; Skorupska et al. 2006). Other mutants that produced diminished amounts of EPS in comparison to the wild type strain, such as *rosR*, formed partially infected nodules that were usually inefficient in nitrogen fixation but plant defense reactions were not observed (Skorupska et al. 2006).

In this work, we isolated non-polar mini-Tn*5* mutants disrupted in *pssA* and *rosR* open reading frames that were defective in EPS production and we confirmed the indispensability of these genes in symbiosis with clover. Both mutants were

complemented by wild type copies of the respective genes and the defective functions of mutants were fully restored. Previously constructed mutants, one with *pssA* disrupted by Tn*5* in the distal promoter region and another with a frame shift mutation in *rosR*, displayed phenotypes similar to those of newly isolated mutants (Janczarek et al. 2001; Janczarek and Skorupska 2007).

The main goal of this work was to assess the possibility of improving the symbiotic potential and competitiveness of *R. leguminosarum* bv. *trifolii* wild type strains by introducing additional copies of *rosR* and *pssA* genes. We have selected five wild type strains that differed on the plasmid and chromosomal levels, and after introducing *pssA* and *rosR* gene copies on broad host range vector, the amount of EPS, growth rates, symbiotic properties and nodulation competitiveness of transconjugants were examined under laboratory conditions.

Irrespective of some differences in growth rates between the particular *R. leguminosarum* bv. *trifolii* field isolates, the presence of multiple copies of *pssA* and especially, *rosR*, enhanced the growth of all tested isolates (Fig. 2). In *Rhizobium etli*, the plasmid carrying *rosR* gene restored the ability of the *rosR* mutant to grow competitively and its prevalence in the rhizosphere was significantly higher than of the wild type strain, suggesting that *rosR* overexpression enhanced growth in the rhizosphere to levels exceeding those of wild type strain (Bittinger et al. 1997).

In our previous study, no visible expression of the *pssA-gusA* fusion in clover nodules was observed (Janczarek and Skorupska 2004). This finding is in good agreement with the results described by Latchford et al. (1991), who found that the *pssA-gusA* fusion was not expressed in bacteroids of *R. leguminosarum* bv. *phaseoli*. It is possible that the sensitivity of the Gus staining method is too low for the detection of *pssA* expression *in planta*. In this work, *pssA* and *rosR* genes were expressed from their own promoters, and, when present in multiple copies in *R. leguminosarum* bv. *trifolii*, substantially increased EPS production and improved symbiosis with clover in relation to the parental strains.

To exclude the possibility that these effects were caused by changes in gene regulation due to the presence of extra copies of promoters, we introduced DNA fragments containing *rosR* and *pssA* promoter regions on pBBR1MCS-2 into the wild type strain Rt24.2. We did not observe any significant differences in the phenotype between Rt24.2 carrying additional copies of *pssA* promoter and the wild type strain, probably because *pssA* encoded enzymatic protein glycosyl-IP-transferase.

However, the introduction of multiple copies of *rosR* promoter into Rt24.2 resulted in some changes in the phenotype that rather resembled the *rosR* mutant phenotype (a decrease of EPS production, nodule number and green mass of clover plants). This opposite effect could be explained by the fact that RosR encoded by a single copy gene might be titrating out in the presence of additional copies of *rosR* promoter (unpublished data). Previously, it was established that RosR bound to the sequence termed the RosR-box located in *rosR* upstream region and slightly influenced its own transcription (Janczarek and Skorupska 2007). But in the presence of multiple copies of *rosR* with a full-length coding region, the amounts of RosR protein and its promoter sequences could be balanced.

Both *pssA* and *rosR* genes encode proteins that affect several cell functions. RosR positively regulates not only *pss* genes (Janczarek and Skorupska 2007) but also positively or negatively regulates several other genes, as was documented by global transcription analysis of *rosR* regulon in *R. etli* (Bittinger and Handelsman 2000). The *rosR* regulon comprises more than fifty genes of different functions, including those involved in polysaccharide production, carbohydrate metabolism and plant infection (Bittinger and Handelsman 2000). Mutation in *pssA* gene affected intracellular levels of 23 proteins in *R. leguminosarum* bv. *trifolii* ANU437 showing pleiotropic function of this gene and the complex regulatory network in the rhizobial cell (Guerreiro et al. 2000; Djordjevic et al. 2003). Overexpression of *pssA* and *rosR* genes probably may activate other genes in several metabolic pathways, among them EPS production, which was increased nearly two times in strains MJ43 and MJ51 harboring multiple copies of *rosR* in comparison to the strains with control plasmid.

When studying the effect of multiple *pssA* and *rosR* gene copies on the symbiotic efficiency of wild type strains, we observed a statistically significant increase in the number of nodules, nodule occupation and fresh and dry shoot mass in all tested strains, in comparison to strains carrying the control plasmid

pBK1 (Table 3). Two approaches were used to study the competitiveness of strains bearing multiple *pssA* and *rosR* gene copies in relation to the wild type strains. When antibiotic selection was applied to differentiate between two strains isolated from clover nodules, we showed that the majority of nodules (up to 80–90%) were occupied by strains carrying additional *pssA* or *rosR* copies (Fig. 4a). When Rt24.2 laboratory strain was tagged with *gusA*, which enabled for the tested strains to be distinguished after nodule staining, the effect of *pssA* and *rosR* genes was significant in the case of Rt24.2 strain but not so visible in the case of field isolates, probably because of the low competitiveness of Rt24.2 strain. Nevertheless, increase in the quantity of nodules invaded by strains carrying multiple copies of *rosR* and *pssA* in comparison to wild type strains, with or without a control plasmid, was observed (Fig. 4b).

In conclusion, we have demonstrated the beneficial effect of increased *pssA* and *rosR* gene copy number on the growth, EPS production, symbiotic efficiency and competitiveness of *R. leguminosarum* bv. *trifolii*. The significance of EPS production in symbiosis and competitiveness could stem from a protective role of this surface polysaccharide during early and late symbiotic stages of rhizobial growth, when rhizobia are subjected to a prolonged oxidative burst from their hosts (Santos et al. 2001). Recently, Davies and Walker (2007) described *Sinorhizobium meliloti* mutants which were both sensitive to oxidative stress in a free-living state as well as symbiotically defective. Several of these mutants were mutated in *exo* genes required for EPS synthesis, indicating that EPS may be involved in the oxidative stress protection in *S. meliloti*. This study contributes to our understanding of the role of EPS in the interaction of rhizobia with their hosts and the environment. Strain modifications that increase EPS production may improve selection of rhizobial inoculants.

Acknowledgments We thank Maria Małek for excellent technical assistance. This work was supported by the grant from the Ministry of Science and Higher Education no. N N303 092234.

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