Regulation of exopolysaccharide production in *Rhizobium leguminosarum* biovar *viciae* WSM710 involves *exoR*

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A mildly acid-sensitive mutant of *Rhizobium leguminosarum* bv. *viciae* WSM710 (WR6-35) produced colonies which were more mucoid in phenotype than the wild-type. Strain WR6-35 contained a single copy of Tn5 and the observed mucoid phenotype, acid sensitivity and Tn5-induced kanamycin resistance were 100% co-transducible using phage RL38. WR6-35 produced threefold more exopolysaccharide (EPS) than the wild-type in minimal medium devoid of a nitrogen source. EPS produced by the mutant and the wild-type was identical as determined by proton NMR spectra. An *EcoRI* rhizobial fragment containing Tn5 and flanking rhizobial sequences was cloned from the mutant, restriction mapped and sequenced. There was extensive similarity between the ORF disrupted by Tn5 in *R. leguminosarum* bv. *viciae* WR6-35 and the *exoR* gene of *Rhizobium* (*Sinorhizobium*) *meliloti* Rm1021 (71.3% identity over 892 bp). At the protein level there was 70% identity and 93.3% similarity over 267 amino acids with the ExoR protein of *R. meliloti* Rm1021. Hydrophilicity profiles of the two proteins from these two rhizobia are superimposable. This gene in *R. leguminosarum* bv. *viciae* was thus designated *exoR*. The data suggest that Tn5 has disrupted a regulatory gene encoding a protein that negatively modulates EPS biosynthesis in *R. leguminosarum* bv. *viciae* WSM710. Despite earlier suggestions that EPS production and acid tolerance might be positively correlated, disruption of *exoR* in either *R. leguminosarum* bv. *viciae* or *R. meliloti* and its associated overproduction of EPS does not result in a more acid-tolerant phenotype than the wild-type when cultures are screened on conventional laboratory agar.

Keywords: exopolysaccharide, acid tolerance, regulator, soil acidity, *Rhizobium*

INTRODUCTION

Root nodule bacteria are able to infect, nodulate, and convert atmospheric N₂ into NH₃ in association with a specific legume host. The symbiotic associations between the root nodule bacteria and legumes are of immense importance since they provide the largest input from biological nitrogen fixation into agricultural production.

Bacterial exopolysaccharide (EPS) is thought to play a

critical role in the rhizobial–plant interaction (Leigh & Coplin, 1992). *Rhizobium* can secrete EPS consisting of either homopolymers (1,2- β -glucans or cellulose) or heteropolysaccharides. The latter are acidic polymers composed of linear arrangements of repeating units containing neutral sugars and uronic acids as well as non-carbohydrate substituents such as acetate, pyruvate, hydroxybutyrate and succinate (Aman *et al.*, 1981; Canter Cremers *et al.*, 1991; Cunningham & Munns, 1984; McNeil *et al.*, 1986).

Mutants of *Rhizobium leguminosarum* defective in EPS production are unable to induce visible nodule development on pea roots (Diebold & Noel, 1989). In contrast, *exo* mutants of *Rhizobium meliloti* (recently renamed *Sinorhizobium meliloti*: De Lajudie *et al.*,

Abbreviation: EPS, exopolysaccharide.

The GenBank accession number for the sequence reported in this paper is L39937.

1994) form nodules on alfalfa, but these are devoid of bacteroids (Finan *et al.*, 1985; Keller *et al.*, 1988; Leigh *et al.*, 1985; Long *et al.*, 1988). Consequently in both these rhizobia acidic heteropolysaccharide is required for successful root invasion and the development of a normal nitrogen-fixing nodule (Finan *et al.*, 1985; Leigh *et al.*, 1985).

EPS synthesis (of *R. leguminosarum* bv. *phaseoli*) and nodulation capability (*R. leguminosarum* bv. *phaseoli* and *R. leguminosarum* bv. *viciae*) are inhibited by multiple copies of a gene termed *psiA* (Borthakur *et al.*, 1985). However, insertional inactivation of *psiA* in *R. leguminosarum* bv. *phaseoli* did not significantly affect EPS synthesis (Borthakur *et al.*, 1985). Inoculation of these inactivated *psiA* strains onto *Phaseolus* roots indicated that although nodules were induced, there was no nitrogen fixation (Borthakur *et al.*, 1985; Borthakur & Johnston, 1987). The *exoX* genes in *R. meliloti* (Reed & Walker, 1991) and *Rhizobium* sp. strain NGR234 (Gray *et al.*, 1990) have similar sequence and functional homology to the *psiA* gene of *R. phaseoli*.

Doherty *et al.* (1988) identified two new loci, *exoR* and *exoS*, involved in the regulation of EPS synthesis in *R. meliloti*. The *exoR* gene product negatively modulates EPS biosynthesis, an effect mediated at the level of gene expression (Reed *et al.*, 1991). A fundamental difference between *exoR* and *psiA* is that the former can inhibit EPS synthesis when present as a single copy in the genome (Doherty *et al.*, 1988). However, *exoR* has not been reported to play a role in EPS biosynthesis in *R. leguminosarum*.

One important stress affecting the *Rhizobium*-legume symbiosis is the progressive acidification of agricultural soils (Coventry & Evans, 1989). Legume pasture productivity significantly decreases as the soil acidifies, due to the acid sensitivity of the prokaryotic symbiont (Munns, 1986; Robson & Loneragan, 1970). The suggestion has been advanced that EPS production might have a protective role, enabling *Rhizobium* strains producing greater amounts of EPS to survive better in conditions of acidic stress than those that produce smaller amounts (Cunningham & Munns, 1984). However, Howieson *et al.* (1988) did not find a strong correlation between polysaccharide production and acid tolerance. These correlations between the amounts of EPS production and acid tolerance have relied on the use of genetically different strains of *Rhizobium* isolated from various regions around the world. The analysis of the acid tolerance of isogenic strains differing only in the extent of EPS production would provide a means of addressing this question directly.

To develop some understanding of the basis of acid tolerance we have generated (Tiwari *et al.*, 1992; O'Hara *et al.*, 1989) and characterized (Goss *et al.*, 1990; Reeve *et al.*, 1993; Tiwari *et al.*, 1996a, b) a number of Tn5-induced acid-sensitive mutants of *R. meliloti* and *R. leguminosarum* bv. *viciae*. During the course of isolating acid-sensitive mutants we discovered a mutant of *R. leguminosarum* bv. *viciae* WSM710 (WR6-35)

which showed a more mucoid phenotype on minimal medium than the parent. The rhizobial DNA flanking each side of Tn5 from the site of insertion was cloned, restriction mapped and sequenced. Evidence is presented which suggests that Tn5 has disrupted a gene encoding a regulatory protein (ExoR) which negatively modulates the biosynthesis of EPS in *R. leguminosarum* bv. *viciae* WSM710.

METHODS

Bacterial strains, plasmids and media. The bacterial strains and plasmids used in this work are shown in Table 1. Strains were stored at -80°C in 15% (v/v) glycerol. Bacterial strains were grown as described by Tiwari *et al.* (1996a), or in the minimal salts medium (MSM) of Brown & Dilworth (1975).

Mutagenesis. The Tn5 mutagenesis procedure of Selveraj & Iyer (1983) was used to isolate the acid-sensitive mutant WR6-35. Acid sensitivity was tested as described earlier (Reeve *et al.*, 1993; Tiwari *et al.*, 1992) by spotting 10^4 cells of the wild-type and the mutant on the same plate.

Transduction. Preparation of phage RL38 stocks and transduction of WSM710 was carried out using the method of Buchanan-Wollaston (1979).

DNA preparation and manipulation. Plasmid and genomic DNA were isolated as described by Tiwari *et al.* (1996a); all other DNA manipulations were performed as described by Sambrook *et al.* (1989). Probe preparation, labelling and hybridization were carried out as described by Tiwari *et al.* (1996a).

DNA sequencing and analysis. DNA sequencing and analysis were as reported by Tiwari *et al.* (1996a). One custom primer was synthesized by Bresatec (5'-CGA CCA TCT GAT GCT GTC-3') and used to obtain double-stranded DNA sequence information of the rhizobial DNA flanking the IS50L.

EPS production. Cells of strains of *R. leguminosarum* bv. *viciae* were grown to mid-exponential phase at pH 7.0 in MSM containing 20 mM mannitol and 10 mM NH_4Cl , washed in minimal salts and resuspended to an OD_{600} of approximately 0.1 in a similar medium, or one lacking NH_4Cl . Cells were incubated at 28°C with shaking and samples of culture harvested at intervals for measurement of cell protein (using a Bio-Rad protein assay kit) and EPS. Cells were removed from culture samples by centrifugation (10 min in a Beckman microfuge E) and EPS was precipitated from the supernatant by adding 0.3 vol. of a 1% hexadecyltrimethylammonium bromide solution stored at 28°C . EPS was centrifuged down (10 min in a Beckman microfuge E) and redissolved in 10% (w/v) NaCl for assay by the anthrone/ H_2SO_4 method (Trevelyan & Harrison, 1952), using glucose in 10% (w/v) NaCl as a standard.

EPS isolation for NMR spectroscopy. Cells were grown in MSM minimal medium and EPS was isolated as described by Doherty *et al.* (1988). Purified EPS samples were dissolved in D_2O (99.8%), freeze-dried and dissolved at a concentration of 15 mg ml^{-1} in D_2O . ^1H -NMR spectra were recorded on a Bruker ARX-500 spectrometer at 50°C . Chemical shifts were referred to chloroform (CDCl_3).

Complementation analysis. The plasmid pM6 containing the *R. meliloti* Rm1021 *exoR* gene (Doherty *et al.*, 1988) was mobilized into *R. leguminosarum* WR6-35 using the helper plasmid pRK2013. Transconjugants were selected on MSM

Table 1. Bacterial strains, phage and plasmids

Strain, phage or plasmid	Relevant characteristics	Source/reference
Strains		
<i>Escherichia coli</i>		
DH5 α	F ⁻ ϕ 80dlacZ Δ M15 <i>recA1 endA1 gyrA96 thi-1 hsdR17</i> ($r_K^- m_K^+$) <i>supE44 relA1 deoR</i> Δ (<i>lacZYA-argF</i>)U169	Bethesda Research Laboratories (1986)
HB101	F ⁻ <i>thi-1 hsdS20</i> ($r_B^- m_B^-$) <i>supE44 recA13 ara-14 leuB6 proA2 lacY1 rpsL20</i> (Sm ^R) <i>xyl-5 ml-1</i>	Boyer & Roulland-Dussoix (1969)
MM294A	<i>pro-82 thi-1 endA1 hsdR17 supE44</i>	G. Walker*
<i>R. leguminosarum</i> bv. <i>viceae</i>		
WSM710	Acid-tolerant strain from <i>Vicia</i> sp. in Japan	J. Howieson†
WR6-35	WSM710 <i>exoR635::Tn5</i>	This study
<i>R. meliloti</i>		
Rm1021	SU47 Sm ^R	Meade <i>et al.</i> (1982)
Rm7095	Rm1021 <i>exoR95::Tn5</i>	Doherty <i>et al.</i> (1988)
Phage		
RL38	Generalized transducing phage	Buchanan-Wollaston (1979)
Plasmids		
pBR322	Cloning vector; Ap ^R Tc ^R	Bolivar <i>et al.</i> (1977)
pGEM-7Zf(+)	Cloning vector; Ap ^R	Promega
pM6	pLAFR1 derivative (<i>exoR</i> ⁺); Tc ^R	Doherty <i>et al.</i> (1988)
pRK2013	Helper plasmid; Km ^R	Ditta <i>et al.</i> (1980)
pWR635	pBR322 containing Km ^R <i>EcoRI</i> fragment of WR6-35	This study
pWR635L	<i>Bam</i> HI- <i>Eco</i> RI fragment of pWR635 containing left inverted repeat of Tn5 and associated rhizobial flanking sequences cloned from pWR635 into pBR322; Ap ^R Km ^R	This study
pWR635R	<i>Hind</i> III fragment of pWR635 containing right inverted repeat of Tn5 and associated rhizobial flanking sequence cloned into pBR322; Ap ^R	This study

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plates containing kanamycin and tetracycline. The ability of plasmid pM6 to complement the *exoR* defect in WR6-35 was assessed by comparing phenotypes of transconjugants against the wild-type on MSM plates.

Nodulation. Seeds of Wirrega pea (*Pisum sativum* L.) or vetch (*Vicia bangalensis* cv. Popany) were surface sterilized with HgCl₂ (0.2%) for 3 min and washed five times with sterile deionized water. Seeds were germinated on TY agar prior to sowing in pots (2 l capacity) containing local yellow (Jandakot) sand which had been steam treated twice at 90 °C for 90 min. Immediately after planting, pea or vetch seedlings were inoculated with either the wild-type WSM710, or the mutant WR6-35. Pots were covered with sterile vermiculite and watered via a side tube to maintain axenic culture. Plants were watered with sterile nutrient solution (Broughton & Dilworth, 1971). Nodules were surface sterilized for 1 min in 70% ethanol, followed by 3 min in 4% sodium hypochlorite and six successive washes in sterile deionized water. Nodules were

crushed in sterile 0.9% NaCl and the contents streaked onto TY plates.

Electron microscopy. Nodule material was fixed overnight at 4 °C in 3% (w/v) glutaraldehyde in 0.025 M phosphate buffer at pH 7.0. The samples were washed several times in 0.025 M phosphate buffer at pH 7.0 before post-fixation in 1% (w/v) osmium tetroxide in 0.025 M phosphate buffer for 2 h at room temperature. After several washes with 0.025 M phosphate buffer (pH 7.0) the samples were left overnight at 4 °C in 1% (w/v) aqueous uranyl acetate solution before dehydration with 30%, 50%, 70%, 90% and finally 100% acetone. Infiltration with Spurr's resin in acetone (Spurr, 1969) from 5% to 90% was accomplished in nine steps, each of 2 h duration at 4 °C; then 100% resin at room temperature for 2 h, before a final wash in 100% resin for 5–8 h also at room temperature. The samples were embedded in fresh Spurr's resin at 60 °C and left for 24 h to ensure complete polymerization. Sections were cut at approximately 90 nm and mounted

on copper grids. Processing and preparation of the samples for electron microscopy was performed by Gordon Thompson at the School of Biological and Environmental Sciences, Murdoch University.

RESULTS AND DISCUSSION

Characteristics of strain WR6-35

Strain WR6-35 is a Tn5-induced mutant which grows on solid JMM salts medium only down to pH 5.0, while the wild-type (WSM710) grows at pH 4.9 (Tiwari *et al.*, 1992); WR6-35 is therefore mildly acid sensitive. In JMM broth culture, the mean generation time for WR6-35 was 3.7 h at pH 7.0, which was comparable to that of the wild-type (3.2 h). On solid MSM with mannitol as the carbon source, colonies of WR6-35 were significantly more mucoid than those of the wild-type.

Transductional analysis, using phage RL38 grown on strain WR6-35 to infect WSM710, showed that the kanamycin resistance, mucoid colony formation and acid-sensitive phenotype were 100% co-transducible. Southern hybridization, using a 5.7 kb *HpaI*–*HpaI* Tn5 probe, showed that WR6-35 contains one copy of Tn5 located in a 17 kb *EcoRI* fragment. A *Bam*HI/*Eco*RI digestion of genomic DNA released two fragments, of 7.0 and 7.4 kb, which hybridized to the probe.

Rhizobial DNA flanking Tn5 was cloned from WR6-35 into the *Eco*RI restriction site of pBR322. This chimaeric plasmid, pWR635, was restriction mapped (Fig. 1a); *Eco*RI digestion generated a 17 kb fragment and *Bam*HI/*Eco*RI digestion generated a 7.0 kb (*Bam*HI rhizobial fragment containing IS50R) fragment and a 7.4 kb (*Bam*HI–*Eco*RI rhizobial fragment containing IS50L) consistent with data from the Southern hybridization.

Sequencing and analysis of the *exoR* gene region

The rhizobial DNA flanking the IS50L or IS50R from the plasmid pWR635 was cloned into pBR322 to create pWR635L or pWR635R, respectively (Fig. 1b). The DNA was further subcloned into pGEM-7Zf(+) using a variety of restriction enzyme sites found within pWR635L and pWR635R. The overall strategy used to completely sequence both strands of the DNA is shown in Fig. 1.

The 1.057 kb DNA fragment around the Tn5 insertion site was sequenced (Fig. 2). Search for a potential ORF using the MacVector analysis program and the universal start codon revealed an ORF spanning the site of Tn5 insertion. This ORF started at position 96 and ended at position 896. It has a putative ribosome-binding site (5'-GAAAGAAA-3') located 9 bp upstream of the ATG initiation codon.

The DNA sequence was then used to search for similarity in the GenBank database using the FASTA algorithm from the programming facility at the University of Georgia (Devereux *et al.*, 1984). The only significant match was with the *exoR* gene of *R. meliloti* (71.3% identity over 892 bp).

The ORF found within the *R. leguminosarum* sequence was converted into its corresponding amino acid sequence (see Fig. 2), which was used to search for any similarity with other protein sequences (using the FASTA algorithm) submitted to the international databases. The protein encoded by this ORF had 70% identity and 93.3% similarity over 267 amino acids with ExoR of *R. meliloti*. Although the calculated pI values for the two ExoR proteins are clearly different (a pI of 7.2 for *R. meliloti* and 5.5 for *R. leguminosarum*), the hydrophilicity profiles, calculated using the Kyte–Doolittle algorithm, reveal that they are very similar over their entire length (Fig. 3). There is thus a strong match at both the DNA and protein level, which suggests that the gene inactivated by Tn5 in WR6-35 is an allele of the *exoR* gene of *R. meliloti*.

Rate of EPS synthesis

The mutant WR6-35 displays a more mucoid phenotype on MSM plates than the wild-type WSM710. To quantify EPS production by the mutant and wild-type and to investigate whether ExoR regulates biosynthesis in *R. leguminosarum* biovar *viciae* as it does in *R. meliloti* (Doherty *et al.*, 1988), we measured the rate of EPS production by cultures of WR6-35 and the wild-type WSM710 incubated with, and without, a nitrogen source (NH₄Cl). The concentration of EPS plotted against the integrated value of protein concentration and time yielded rates of EPS synthesis per unit protein per unit time (Fig. 4). In the absence of 10 mM NH₄Cl, WR6-35 produced threefold more EPS than the wild-type; in the presence of NH₄Cl, the mutant produced only 1.3 times the wild-type amount.

The behaviour of the *exoR* mutants of *R. meliloti* Rm1021 and *R. leguminosarum* bv. *viciae* WSM710 is compared in Table 2. The units of EPS are arbitrary; they are derived from the actual rates of EPS synthesis for the two *R. leguminosarum* strains and the reported (Doherty *et al.*, 1988) amount of EPS at time of harvest for the two strains of *R. meliloti*. *R. meliloti* Rm7095 produced 220-fold more EPS than the wild-type *R. meliloti* Rm1021 in the presence of ammonia. The large amount of EPS produced by the *R. meliloti* mutant (Doherty *et al.*, 1988) was essentially independent of the presence or absence of ammonia. EPS produced by the *R. leguminosarum* mutant also was not affected by the presence or absence of ammonia (Table 2). However, the two wild-type organisms appear to respond quite differently to ammonia addition; it stimulates the rate of EPS production about 3-fold for *R. leguminosarum* WSM710 but decreases EPS production by a factor of 30-fold in *R. meliloti* Rm1021. A mutation of *exoR* in both organisms therefore results in increased EPS production which is no longer affected by ammonia.

Characterization of EPS

A mutation in *exoR* would be expected to alter the amount, but not the type, of EPS produced. The ¹H-NMR spectra of the EPS produced by WSM710 and

Fig. 1. Restriction maps of pWR635 and subclones used in this study. (a) A 17 kb *EcoRI* fragment containing Tn5 was cloned from *R. leguminosarum* WR6-35 into the *EcoRI* site of pBR322 to construct pWR635. (b) The left or right flanking rhizobial sequences derived from pWR635 were cloned into pBR322 to construct pWR635L or pWR635R, respectively (see Table 1 for a detailed description). These two clones were restriction mapped in further detail to provide sites suitable for generating additional subclones. Sequence data from the various subclones was generated using M13 forward and reverse primers (—) and custom-synthesized primers (---). A filled circle on a dashed line represents the use of Tn5 primer on clones pWR635L and pWR635R. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III; Hp, *Hpa*I; Ks, *Ksp*I; P, *Pst*I; X, *Xho*I.

<i>KspI</i>	-CC GCG GAT CGC TCG CTT CGA AGG AAT ACA ATT CCG GGA TGC AGG GCG CGA GCG CGG TTG	60
	CCG CCG GCG TGA GAT GAA CGA BAG AAA TCG GTT TAA ATG GTG ACG TCC GAG TTC AAA CTG	120
		M V T S E F K L
	TTC AAA TTC ATG CTG ATG GGC ATG TCG ATA GCC GTC GCG CTG CCG TCC GGT CCG TGC	180
	F X F M L M G M S I A V A L A L S G P C	
	CGC GCA TTC GAT ATC AAA GGC GGC GTC AGC AAG GAA TCC GGA CCC TTC GAC CTC TTC AAG	240
	R A F D I K G G V S K E S G P F D L F K	
	TTC GGC TTC AAG GGC TAC AAG AAC GGC CAG AAG GAA GAG GCG GTG GAA GGC TAT AGA TAC	300
	F G F K A Y K N G Q K E A V E A Y R Y	
	GCC GCC GAA AAG GGC CAC ACC GGC TCG CCG TGG GCG CTT GCC AAC ATG TAT GCC GAT GGC	360
	A A E K G H T G S R W A L A N M Y A D G	
	GAC GGC GTC ACC CAG GAT GAT TTC GAA GGC CTC AAG ATC TAT ACC GAG ATC GCC CAG CAG	420
	D G V T Q D F F E A F K I Y S E I A L F	
	GGC GTC GAG CCC GGT TCG GAA GAT ACC GGC TTC TTC GTC AAC GCG CTC TCG CTC GGC	480
	G V E P G S E D T G F F V N A L L S L A	
	AAC TAT TAC AAG CAC GGC ACT GCC GGC AGC CCG GTC AGG ATC GAC CTC AGC CAG GCA GCG	540
	N Y Y K H G I A G S P V R I D L S Q A R	
	CAA CTT TAT TTC CAG GTG GGC TCC ACC TTT GGC GTC CCC GAA GCG CAG TTC CAA CTC GCG	600
	Q L Y F T Q V A S T P G V F E A Q F Q L A	
	CAG ATG ATG CTC GGC GAG GGC GGC AAT GCC AGC CCG CAG CAG GCG AAG AAA TGG TTG	660
	Q M M L A G E G N A S P Q Q A K K W L	
	NAC CAG GGC CCG AAG AGC GGC CAT CCC GGC GGC ATG GCG GTC TTC GGC AAT ATC CTC TTC	720
	N Q A R K S G H P G A M A V F G N I L F	
	GAC GAA GGC CAG ACG GGC CGT GGT CTT GCG CTG ATG ACG GCG GCA CTC GAC CCG TGC AAG	780
	A L E G Q T A R G L A L M T A A L D R C K	
	CGC AAG GAC TGT GGC TGG ATG GAA GCG TTG CAG GAG CAG GCC TTC TCT GTT GCG AAC GAG	840
	P K D C G W M E A L Q E Q A F S V A N E	
	GCC GAC GCG CCG ACG GCG GTA TCC CTC TCG CAC AGC ATC GCG ACC GGT TCC GAA CTC GCG	900
	A D R K R T A V S L S H S I A T G S D D	
	AGG AGT GCC GTG CAT CCT TTC GGA CCC ACG GCA CCG TAA GGC GAA GAA GGC TCA GGC TGC	960
	AAA ATC GAA ATA GGC GAT AAC AGG CAC GTG GTC GGA CCG CTT TTC CCA AGC CCG CAG ATG	1020
	TTT CTC GAT CCG GGC CGA CGT CAT CCG GTC GGC GGC TT	

Fig. 2. Nucleotide and annotated amino acid sequence of the 1.057 kb DNA fragment containing the *exoR* gene of *R. leguminosarum* WSM419. A potential ribosome-binding site is underlined. The boxed region represents the 9 bp duplicated by Tn5 integration.

WR6-35 are superimposable (Fig. 5a, b), providing evidence that no modification of the EPS has occurred. The $^1\text{H-NMR}$ spectra of the repeating units of the acidic heteropolysaccharide produced by strain WSM710 or WR6-35 were very similar to those produced by other strains of *R. leguminosarum* (McNeil *et al.*, 1986). These other EPSs consist of branched octasaccharide repeating units bearing pyruvyl-, *O*-acetyl and 3-hydroxybutanoyl substitutions in amounts which vary slightly between strains (Leigh & Coplin, 1992). Similar

Fig. 3. Hydrophilicity analysis of the predicted amino acid sequence of the ExoR protein of *R. meliloti* (a) and *R. leguminosarum* (b) using the MacVector Kyte-Doolittle algorithm and a window size of 7. Positive values indicate hydrophilic regions; negative values indicate hydrophobic regions.

substitutions on the EPS of WSM710 and WR6-35 are evidenced by the resonances at δ 1.35–1.45 p.p.m. (pyruvyl CH_3), δ 2.1–2.2 p.p.m. (acetyl CH_3), δ 1.3 p.p.m. (3-hydroxybutanoyl CH_3) and δ 2.65 p.p.m. (3-hydroxybutanoyl CH_2).

Symbiotic phenotype

Inoculation of Wirrega pea with WSM710 or WR6-35 resulted in the formation of mostly red nodules with a minority of small white nodules. Bacteria isolated from red or white nodules on plants inoculated with WR6-35 were kanamycin resistant and showed the mutant mucoid phenotype in all cases. Southern hybridization

Fig. 4. Rate of EPS production in MSM medium by *R. leguminosarum* WSM710 (filled symbols) and its Tn5-induced mutant WR6-35 (open symbols) in the presence (triangles) and absence (circles) of NH_4Cl .

Table 2. Comparison of EPS production by *R. meliloti* Rm1021, *R. leguminosarum* WSM710 and the *exoR* mutants Rm7095 and WR6-35 in the presence (+ NH_4Cl) and absence (– NH_4Cl) of a nitrogen source

	<i>R. meliloti</i> *		<i>R. leguminosarum</i> bv. <i>viciae</i> †	
	Rm1021	Rm7095	WSM710	WR6-35
+ NH_4Cl	0.05	11.2	9.92	12.8
– NH_4Cl	1.16	12.8	3.56	9.98

* Values [mg anthrone-positive material (mg protein) $^{-1}$] presented for *R. meliloti* are those published by Donovan *et al.* (1988).

† Relative values of EPS production for *R. leguminosarum* have been calculated by scaling the rates of synthesis [mg anthrone positive material min^{-1} (mg protein) $^{-1}$] such that the figure of maximum EPS production for *R. leguminosarum* matches the maximum figure of EPS production of *R. meliloti* (12.8).

of a *Bam*HI/*Eco*RI digest of genomic DNA from clones derived from red or white nodules with Tn5 as a probe showed that the transposon remained inserted in *exoR*. Transmission electron microscopy of thin sections of red or white nodules from plants inoculated with WSM710 or WR6-35 revealed normal bacteroid development within both white and pink nodules.

The results indicate that the nodule formation resulting from inoculation of plants with the mutant WR6-35 is not caused by either a reversion or suppression of EPS production by an extragenic mutation.

This contrasts with the behaviour of the *exoR* mutant of *R. meliloti*: some plants produced white nodules (with no bacteroids) and some had pink nodules (possibly containing pseudo-revertants that acquired mutations

Fig. 5. ^1H -NMR spectra of EPS produced by *R. leguminosarum* bv. *viciae* WSM710 (a) and WR6-35 (b). The assignments are as follows: ac, methyl protons of the acetyl groups; es, methylene protons of the 3-hydroxybutanoic ester; HDO, hemideuterated water; pyr, methyl protons of the 1-carboxyethylidene (pyruvate) groups; ring, ring protons of the sugars; sa, methyl protons of sodium acetate (a contaminant of repeating units; McNeil *et al.*, 1986); ss, spinning side bands of the solvent peak.

suppressing the mucoid phenotype) and others produced both pink and white nodules (Doherty *et al.*, 1988).

WR6-35 shows the same nodulation pattern on vetch (*Vicia bengalensis* cv. Popany) as the wild-type.

Complementation analysis

When the plasmid pM6 containing the *exoR* gene from *R. meliloti* Rm1021 was mobilized into WR6-35, the transconjugants still had the mutant mucoid phenotype.

The *exoR* gene of *R. meliloti* does not complement the *exoR* defect of *R. leguminosarum* because either the ExoR protein is functionally different or it is not being expressed in this background.

Test for acid sensitivity of an EPS-overproducing mutant of *R. meliloti*

The acid sensitivity of the wild-type Rm1021 and the *exoR* mutant Rm7095 was compared by spotting 10^4 stationary-phase cells onto JMM plates at pH 5.6. After 5 d incubation at 28 °C there was visible growth of the wild-type strain, but no growth of the mutant. Thus, the observed acid-sensitive phenotype has been found in two different species of *Rhizobium* which contain an insertionally inactivated *exoR* gene. Both *exoR* mutants, however, showed visible growth on low-pH plates if incubated for 2 weeks at 28 °C.

Concluding remarks

The data presented in this paper provide evidence that the *exoR* gene of *R. leguminosarum* bv. *viciae* WSM710 regulates EPS biosynthesis, though in a significantly less profound manner than in *R. meliloti*.

A mutation in *exoR* caused an apparent acid-sensitivity in both *R. leguminosarum* and *R. meliloti*. Since the change in acid tolerance between the wild-type and mutant is small, the growth retardation of an *exoR* mutant at low pH may be linked to perturbations in cell processes resulting from increased EPS biosynthesis. Alternatively, extra EPS synthesis, an energy-consuming process, in combination with acidity might act as a double stress resulting in an acid-sensitive phenotype.

Cunningham & Munns (1984) reported a positive correlation between acid tolerance and EPS production using a wide variety of non-isogenic strains of *R. meliloti*. In this report we have used isogenic strains of *R. leguminosarum* and *R. meliloti* overproducing EPS and have shown that the production of more EPS does not result in improved acid tolerance.

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