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 N\textsubscript{2} into NH\textsubscript{3} 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-\(\beta\)-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.*

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**Abbreviation:** EPS, exopolysaccharide.

The GenBank accession number for the sequence reported in this paper is L39937.
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 nodule 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). 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 10⁵ 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 ISSOL.

**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 NH₄Cl, washed in minimal salts and resuspended to an OD₆₀₀ of approximately 0.1 in a similar medium, or one lacking NH₄Cl. 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/ _H₂SO₄_ method (Trevelyan & Harrison, 1932), 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. (1998). Purified EPS samples were dissolved in _D₂O_ (99.8%), freeze-dried and dissolved at a concentration of 15 mg ml⁻¹ in _D₂O_. ¹H-NMR spectra were recorded on a Bruker ARX-500 spectrometer at 50 °C. Chemical shifts were referred to chloroform (CDCl₃).

**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

<table>
<thead>
<tr>
<th>Strain, phage or plasmid</th>
<th>Relevant characteristics</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli DH5α</td>
<td>F− g80lacZAM15 recA1 endA1 gyrA96 thi-1 hsdR17 (rK mλ) supE44 relA1 deoR Δ(lacZYA−argF)U169</td>
<td>Bethesda Research Laboratories (1986)</td>
</tr>
<tr>
<td>HB101</td>
<td>F− thi-1 hsdS20 (rK mλ) supE44 recA13 ara-14 leuB6 proA2 lacY1 rpsL20 (SmR) xyl-5 mil-1 pro-82 thi-1 endA1 hsdR17 supE44</td>
<td>Boyer &amp; Roulland-Dussoix (1969)</td>
</tr>
<tr>
<td>MM294A</td>
<td></td>
<td>G. Walker*</td>
</tr>
<tr>
<td>R. leguminosarum bv. viciae WSM710</td>
<td>Acid-tolerant strain from Vicia sp. in Japan</td>
<td>J. Howieson†</td>
</tr>
<tr>
<td>WR6-35</td>
<td>exoR635::Tn5</td>
<td>This study</td>
</tr>
<tr>
<td>R. meliloti Rm1021</td>
<td>SU47 SmR</td>
<td>Meade et al. (1982)</td>
</tr>
<tr>
<td>Rm7095</td>
<td>exoR95::Tn5</td>
<td>Doherty et al. (1988)</td>
</tr>
<tr>
<td>Phage RL38</td>
<td>Generalized transducing phage</td>
<td>Buchanan-Wollaston (1979)</td>
</tr>
<tr>
<td>Plasmids pBR322</td>
<td>Cloning vector; ApR TcR</td>
<td>Bolivar et al. (1977)</td>
</tr>
<tr>
<td>pGEM-7Zf(+)</td>
<td>Cloning vector; ApR</td>
<td>Promega</td>
</tr>
<tr>
<td>pM6</td>
<td>pLAFl derivative (exoR'); TcR</td>
<td>Doherty et al. (1988)</td>
</tr>
<tr>
<td>pkK2013</td>
<td>Helper plasmid; KmR</td>
<td>Ditta et al. (1980)</td>
</tr>
<tr>
<td>pWR635</td>
<td>pBR322 containing KmR EcoRI fragment of WR6-35</td>
<td>This study</td>
</tr>
<tr>
<td>pWR635L</td>
<td>BamHI−EcoRI fragment of pWR635 containing left inverted repeat of Tn5 and associated rhizobial flanking sequences cloned from pWR635 into pBR322; ApR KmR</td>
<td>This study</td>
</tr>
<tr>
<td>pWR635R</td>
<td>HindIII fragment of pWR635 containing right inverted repeat of Tn5 and associated rhizobial flanking sequence cloned into pBR322; ApR</td>
<td>This study</td>
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</table>

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† Centre for Legumes in Mediterranean Agriculture, University of Western Australia.

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 sativa L. 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 probe, showed that WR6-35 contains one copy of Tn5 located in a 17 kb EcoRI fragment. A BamHI/EcoRI 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 EcoRI restriction site of pBR322. This chimaeric plasmid, pWR635, was restriction mapped (Fig. 1a); EcoRI digestion generated a 17 kb fragment and BamHI/EcoRI digestion generated a 7.0 kb (BamHI rhizobial fragment containing IS50R) and a 7.4 kb (BamHI–EcoRI 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
Tn5 integration.

WR6-35 were very similar to those produced by other strains of *R. leguminosarum* (McNeil et al., 1986). These other EPSs consist of branched ocrasaccharide repeating units bearing pyruvyl-, O-acetyl and 3-hydroxybutanoyl substitutions in amounts which vary slightly between strains (Leigh & Coplin, 1992). Similar substitutions on the EPS of WSM710 and WR6-35 are evidenced by the resonances at δ 1.35-1.45 p.p.m. (pyruvyl CH₂), δ 2.1-2.2 p.p.m. (acetyl CH₃), δ 1.5 p.p.m. (3-hydroxybutanoyl CH₂) and δ 2.65 p.p.m. (3-hydroxybutanoyl CH₃).

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

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**Fig. 1.** Restriction maps of pWR635 and subclones used in this study. (a) A 17 kb EcoRI fragment containing Tn5 was cloned 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, BamHI; Bg, BgIII; E, EcoRI; H, HindIII; Hp, HpaI; Ks, KspI; P, PstI; X, Xhol.

**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.

**Fig. 3.** Hydrophilicity analysis of the predicted amino acid sequence of the *exoR* protein of *R. melliloti* (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.
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 NH₄Cl.

Table 2. Comparison of EPS production by R. meliloti Rm1021, R. leguminosarum WSM710 and the exoR mutants Rm7095 and WR6-35 in the presence (+ NH₄Cl) and absence (− NH₄Cl) of a nitrogen source

<table>
<thead>
<tr>
<th>R. meliloti*</th>
<th>R. leguminosarum bv. viciae†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WSM710</td>
</tr>
<tr>
<td>+ NH₄Cl</td>
<td>0.05</td>
</tr>
<tr>
<td>− NH₄Cl</td>
<td>1.16</td>
</tr>
</tbody>
</table>

* 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 BamHI/EcoRI 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 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 bangalensis 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 10² 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 acid-sensitive phenotype has been found in two different species of *Rhizobium* which contain an insertional 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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