Structure and role in symbiosis of the *exoB* gene of *Rhizobium leguminosarum* bv *trifolii*

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**Abstract** The *Rhizobium leguminosarum* bv *trifolii* *exoB* gene has been isolated by heterologous complementation of an *exoB* mutant of *R. meliloti*. We have cloned a chromosomal DNA fragment from the *R. leguminosarum* bv *trifolii* genome that contains an open reading frame of 981 bp showing 80% identity at the amino acid level to the UDP-glucose 4-epimerase of *R. meliloti*. This enzyme produces UDP-galactose, the donor of galactosyl residues for the lipid-linked oligosaccharide repeat units of various heteropolysaccharides of rhizobia. An *R. leguminosarum* bv *trifolii* *exoB* disruption mutant differed from the wild type in the structure of both the acidic exopolysaccharide and the lipopolysaccharide. The acidic exopolysaccharide made by our wild-type strain is similar to the Type 2 exopolysaccharide made by other *R. leguminosarum* bv *trifolii* wild types. The exopolysaccharide made by the *exoB* mutant lacked the galactose residue and the substitutions attached to it. The *exoB* mutant induced the development of abnormal root nodules and was almost completely unable to invade plant cells. Our results stress the importance of *exoB* in the *Rhizobium*-plant interaction.

**Key words** *Rhizobium leguminosarum* bv *trifolii* · *Trifolium subterraneum* · *Trifolium repens* · *exoB* gene · Exopolysaccharide

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**Introduction**

The extracellular and surface polysaccharides produced by *Rhizobium* constitute the interface between the bacterial cell and its environment and are involved in the interactions leading to the establishment of an effective *Rhizobium*-legume symbiosis (Coronado et al. 1996). These polysaccharides include extracellular heteropolysaccharides (EPS), capsular polysaccharides, and lipopolysaccharides (LPS) of the outer membrane. Cyclic (1→2)-β-D glucan is found in the periplasm and culture medium of some strains.

*Rhizobium meliloti* synthesizes two different heteropolysaccharides. EPSI is a succinoglycan necessary for the invasion of alfalfa root nodules; EPSI-deficient mutants fail to form infection threads. EPSII is a galactoglucan with a completely different structure; EPSII-deficient mutants are still able to form an effective symbiosis with alfalfa. Twenty-two *exo* genes for EPSI synthesis have been identified in *R. meliloti* Rm2011; 19 of them reside in a contiguous 24-kb cluster on the second symbiotic megaplasmid (pSymb) (Leigh and Walker 1994).

The *R. meliloti* *exoB* gene encodes a UDP-glucose 4-epimerase (Buendía et al. 1991) that converts UDP-glucose into UDP-galactose. Since galactose is a common component of both EPS and LPS (Diebold and Noel 1989; Bhagwat et al. 1991), *exoB* mutants should be pleiotropic and defective in the synthesis of both polysaccharides. The symbiotic behavior of *exoB* mutants depends on both the *Rhizobium* and the legume host species. An *exoB* mutant of *R. meliloti* induces empty, ineffective nodules on alfalfa (Leigh and Lee 1988). An *exoB* mutant of *Rhizobium* sp. GRH2 induces a few empty nodules on *Acacia* and fails to nodulate most herbaceous legume hosts (Lopez-Lara et al. 1995). *R. leguminosarum* bv *viciae* *exoB* mutants produce only some root hair deformation and rare, abortive infection threads on *Vicia villosa* (Canter Cremers et al. 1990). In contrast, a *Rhizobium* sp. (Ollero et al. 1994) *exoB* mu-
tant induces effective, indeterminate nodules on the host, *Hedysarum coronarium*.

Strains of *R. leguminosarum* bv *trifolii* may be classified into two major groups and one minor group according to the stoichiometry and the linkage site of the non-carbohydrate substituents in EPS (Philip-Hollingsworth et al. 1989). The symbiotic behavior of some *exo* mutants of *R. leguminosarum* bv *trifolii* has been described, but they have not been characterized at the genetic level. Spontaneous and Tn5-induced *exo* mutants of different strains of *R. leguminosarum* bv *trifolii* induce small, white, and ineffective nodules on clover roots (Rolfe et al. 1980; Chakravorty et al. 1982; Derylo et al. 1986). These nodules contain infection threads, but few of the plant cells contain intracellular bacteria.

We have constructed an *exoB* disruption mutant of *R. leguminosarum* bv *trifolii* to determine the role of this gene in EPS structure and symbiotic behavior.

### Materials and methods

**Strains, plasmids, and media**

Strains and plasmids used in this study are listed in Table 1. *R. meliloti* strains were grown in rich medium YTA (O’Toole et al. 1973) or LB medium (Ausubel et al. 1989) supplemented with 2.5 mM MgSO₄ and 2.5 mM CaCl₂ at 30°C. *R. leguminosarum* bv *trifolii* strains were grown in YT medium (Beringer 1974) or defined BII agar medium (Dazzo 1982) at 30°C. For Congo red absorption, bacteria were streaked on plates of YEMCR agar (Zevenhuizen et al. 1986). *Escherichia coli* strains were grown in LB medium at 37°C. When appropriate, media were supplemented with 200 µg/ml neomycin, 10 µg/ml nalidixic acid, 50 µg/ml kanamycin, 100 µg/ml ampicillin, 20 µg/ml gentamicin, 100 µg/ml spectinomycin, 5 µg/ml tetracycline for *Rhizobium* strains and 15 µg/ml for *E. coli* strains or 200 µg/ml Calcofluor white M2R (Fluorescent Brightener 28, Sigma, St. Louis, Mo., USA).

An *R. leguminosarum* bv *trifolii* gene bank, previously constructed in *E. coli* HB101 (Rodriguez-Quiñones et al. 1989), was used for the heterologous complementation of the *exoB* mutant, *R. meliloti* 7094. Cosmid pVK102 derivatives were transferred conjugatively in triparental matings as described by Ditta (1986).

**DNA manipulations and analysis**

*E. coli* plasmid DNA was isolated by alkaline lysis (Ausubel et al. 1989). *Rhizobium* plasmid DNA was isolated according to Ish-Horowicz and Burke (1981). Bacterial genomic DNA was isolated by selective precipitation with CTAB (hexadecyltrimethyl ammonium bromide) as described by Ausubel et al. (1989). Plasmid characterization was performed according to the procedure of Eckhardt (1978) as modified by Rosenberg et al. (1982). DNA was digested, electrophoresed in 0.7% agarose gels, and blotted onto nylon membranes (Hybond-N, Amersham, Buckinghamshire, UK, 0.45 µm) according to the Southern blotting procedure. DNA probes were prepared using the Multiprime DNA labelling system (Amersham). Hybridizations were performed overnight at 37°C in 5 x SSC, 50% formamide, 25 mM NaH₂PO₄, 25 mM Na₂HPO₄, 5 x Denhardt’s solution, containing 500 µg/ml of herring sperm DNA (Kondorosi et al. 1982). Tn5 insertions in plasmid pBSB43 were physically mapped as described by Ditta (1986). DNA sequences were obtained from overlapping nested deletion clones generated by exonuclease III digestion according to Henikoff (1984). Sequencing reactions were performed using the Auto-Read Sequencing kit (Pharmacia Biotech, Uppsala, Sweden). Sequence data were obtained and processed using the A.L.F. DNA Sequencer (Pharmacia Biotech) according to the manufacturer’s instructions. The nucleotide and amino acid sequences were analyzed using the BESTFIT program of the Genetics Computer Group (GCG) program package of the University of Wisconsin (Devereux et al. 1984).

### Plant nodulation

*Rhizobium* strains were assayed for their symbiotic phenotypes on alfalfa (*Medicago sativa cv Moapa*) and clover plants (*Trifolium repens cv Huita* and *T. subterraneum cv Seaton Park*) grown in Leonard jars, and analyzed 4–5 weeks after inoculation, (Corbin et al. 1982). For each strain, at least six nodules from different plants were collected. Nodules were fixed for 2 h at 4°C with glutaraldehyde (30 ml/l in 100 mM sodium cacodylate, pH 7.2). After two rinses in this buffer, the nodules were postfixed for 2 h in OsO₄ (10 g/l in 100 mM sodium cacodylate, pH 7.2). After three further rinses, the samples were dehydrated in a graded acetone series at 4°C. Samples were maintained for 2 h in uranyl acetate (10 g/l in 70% acetone) before complete dehydration. Finally, nodules were embedded in Spurr medium. Sections were examined by combined light and transmission electron microscopy following staining with (1 g/l) toluidine blue or lead citrate/uranyl acetate, respectively.

### Exopolysaccharide analysis

Bacteria were grown in shaker flasks containing 500 ml of BIII medium at 30°C for 5 days to a density of 0.6 OD₆₀₀. After removing the bacteria by centrifugation, two volumes of cold ethanol were added to the supernatant. The EPS precipitate was collected by centrifugation, lyophilized, redissolved in distilled water, and dialyzed (12 000–14 000 molecular weight cutoff) against 2 l of distilled water at 4°C with five to seven changes over a 3-day period to remove the majority of the β-1,2 glucans and other oligomers. Finally, the EPS solution was lyophilized. The EPS were analyzed by proton nuclear magnetic resonance (¹H-NMR) spectroscopy and combined gas-liquid chromatography/mass spectrometry (GLC/MS). For ¹H-NMR analysis, 3 mg of purified EPS were dissolved in deuterium oxide (99.7%, 0.4 ml), lyophilized, redissolved in deuterium oxide (99.99%, 0.4 ml), and sonicated for 1 min at room temperature. Spectra were recorded at 500 MHz and 70°C with presaturation of the solvent peak at δ 4.25. For GLC/MS analysis of glycosyl compositions, EPS samples were acid-hydrolyzed and the released sugars converted to their corresponding alditol acetate derivatives as previously described (Hollingsworth et al. 1984). Uronic acids were identified by carboxyl reduction with sodium borodeuteride followed by GLC/MS analysis of the alditol acetate derivatives with selected ion monitoring for 217 a.m.u. and 219 a.m.u. as previously described (Hollingsworth et al. 1984; Philip-Hollingsworth et al. 1989).

Electrophoresis of lipopolysaccharides

For SDS-polyacrylamide gel electrophoresis, the LPS were solubilized from proteinase K-treated cells and electrophoresed in 16.5% polyacrylamide gels as described by Köpflin et al. (1993). Gels were fixed and silver-stained as described by Kittelberger and Hilbink (1993). LPS from the smooth strain *Salmonella typhi- munium* and the rough strains *S. typhimurium* TV119 and SL1181, purchased from Sigma, were used as standards.

### Results

A gene from *R. leguminosarum* bv *trifolii* homologous to the *R. meliloti* *exoB* gene

Ten exconjugants of the *exoB* mutant, *R. meliloti* 7094, carrying a cosmid from the *R. leguminosarum* bv *trifolii*
gene bank, were examined for their capacity to form fluorescent colonies on Calcofluor LB media. Physical studies showed that all ten *Rhizobium* clones analyzed were identical. The cosmid, pBSB4, which restored colony fluorescence to *R. meliloti* 7094, was chosen. This cosmid did not complement *R. meliloti* exoA, exoF or exoM mutants.

Hybridization with an internal fragment of the *R. meliloti* exoB gene contained in cosmid pD2 revealed that the cosmid pBSB4 contained a 3.7-kb *PstI* fragment and a 2.2-kb *BamHI* fragment that hybridized to the probe. These fragments were subcloned in pRK404 to obtain plasmids pBSB43 and pBSB42, respectively. Plasmid pBSB43 complemented *R. meliloti* 7094, allowing formation of fluorescent colonies on Calcofluor LB agar and effective nodulation of alfalfa, but plasmid pBSB42 did not complement any of the mutant phenotypes. These results of hybridization and complementa-

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
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<tr>
<td><strong>Rhizobium strains</strong></td>
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<tr>
<td>R. leguminosarum bv trifolii</td>
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<tr>
<td>RS800</td>
<td>Wild type, Nal&lt;sup&gt;f&lt;/sup&gt;</td>
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<td>RS12</td>
<td><em>exoB</em> mutant RS800 derivative, Sp&lt;sup&gt;f&lt;/sup&gt;</td>
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<td><strong>R. meliloti</strong></td>
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<td>Rm1021</td>
<td>Sm&lt;sup&gt;f&lt;/sup&gt;, SU47 derivative</td>
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<td>Rm7094</td>
<td>Rm1021 <em>exoB</em>:Tn5</td>
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<td>Rm7031</td>
<td>Rm1021 <em>exoA</em>:Tn5</td>
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<td>Rm1021 <em>exoF</em>:Tn5</td>
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<td>Rm8457</td>
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<td><strong>Escherichia coli strains</strong></td>
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<td>HB101</td>
<td><em>hsdS, hsdM, pro, leu, thi, gal, lacY&lt;sup&gt;f&lt;/sup&gt;, recA&lt;sup&gt;f&lt;/sup&gt;, Sm&lt;sup&gt;f&lt;/sup&gt;</em>,</td>
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<td>JM109</td>
<td>*recA&lt;sup&gt;1&lt;/sup&gt;, endA&lt;sup&gt;1&lt;/sup&gt;, gyrA&lt;sup&gt;96&lt;/sup&gt;, thi, hsdR17, supE44, relA&lt;sup&gt;1&lt;/sup&gt;, Δ(lac-proAB) F&lt;sup&gt;n&lt;/sup&gt; (traD36, proAB, lacI&lt;sup&gt;q&lt;/sup&gt;Z&lt;sup&gt;D&lt;/sup&gt;M15)</td>
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<td>S17-1</td>
<td>F&lt;sup&gt;f&lt;/sup&gt;, <em>recA, hsdR, RP4-2</em></td>
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<td>C2110</td>
<td><em>polA&lt;sup&gt;1&lt;/sup&gt;, rha, his, Nal&lt;sup&gt;f&lt;/sup&gt;, Rif&lt;sup&gt;f&lt;/sup&gt;</em>,</td>
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<td>HB101::Tn5</td>
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<td><strong>Plasmids</strong></td>
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<td>pKK2073</td>
<td>“Helper” plasmid, Sp&lt;sup&gt;f&lt;/sup&gt;</td>
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<td>pVK102, gene bank</td>
<td><em>R. leguminosarum</em> bv <em>trifolii</em> genomic DNA <em>HindIII</em> fragments cloned in pVK102, Te&lt;sup&gt;f&lt;/sup&gt;</td>
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<td>pD2</td>
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<td>pRK404</td>
<td>pRK290 derivative, Te&lt;sup&gt;f&lt;/sup&gt;</td>
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<td>pUT miniTn5 Sp/Sm</td>
<td>pUT derivative containing the miniTn5 Sp/Sm element</td>
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<td>pJQ200SK</td>
<td>pACYC184 derivative, Gm&lt;sup&gt;f&lt;/sup&gt;</td>
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<td>pBSB4</td>
<td>Cosmid containing <em>exoB</em> gene of RS800</td>
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<td>pBSB43</td>
<td>pKK404 derivative containing the 3.7-kb <em>PstI</em> fragment DNA of RS800</td>
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<td>pBSB42</td>
<td>pRK404 derivative containing the 2.2-kb <em>BamHI</em> fragment DNA of RS800</td>
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<td>pBSB43-3</td>
<td>pBSB43 derivative containing a Tn5 insertion</td>
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<td>pBSB43-4</td>
<td>pBSB43 derivative containing a Tn5 insertion</td>
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<td>pBSB43-6</td>
<td>pBSB43 derivative containing a Tn5 insertion</td>
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<td>pBSB43-8</td>
<td>pBSB43 derivative containing a Tn5 insertion</td>
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<tr>
<td>pJQ2003</td>
<td>pJQ200SK derivative containing the RS800 <em>exoB</em> gene disrupted by the Sp/Sm interposon</td>
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Table 1 Strains and plasmids (*antibiotic resistance, Nal nalidixic acid, Sp spectinomycin, Sm streptomycin, Te tetracycline, Km kanamycin, Rif rifampicin, Gm gentamicin*)
tion studies suggested that an *R. leguminosarum* bv trifolii *exoB* homolog, which contains a *Bam*HI restriction site, is located on the 3.7-kb *Pst*I fragment.

*R. leguminosarum* bv trifolii RS800 harbors three plasmids with molecular weights of about 190, 280, and 470 MDa (Rodriguez-Quinones et al. 1989). In order to locate the *exoB* gene in the genome of RS800, a blotted Eckhardt gel (Rosenberg et al. 1982) was hybridized using the 3.7-kb *Pst*I fragment as a probe. This fragment hybridized with the second symbiotic megaplasmid of *R. meliloti* 1021 and with chromosomal DNA of RS800, but not with plasmid DNA of RS800. This confirmed the plasmid location of gene *exoB* in *R. meliloti* and showed the chromosomal location of the homologous gene of *R. leguminosarum* bv trifolii.

The 2-kb EcoRV-*Pst*I fragment internal to the 3.7-kb *Pst*I fragment was sequenced. It contained an open reading frame from the only ATG codon at position 210 to the TGA codon at position 1191 (Fig. 1). A potential ribosome-binding site (AGGG) was identified just 6 bp upstream of the putative start codon. The deduced polypeptide product was 327 amino acids in length and showed 80.0% identity and 89.2% similarity to the deduced amino acid sequence of the UDP-glucose 4-epimerase of *E. coli* (Lemaire and Müller-Hill 1986). The 2-kb *EcoRV*- *Pst*I fragment was sequenced. It contained an open reading frame from the only ATG codon at position 210 to the TGA codon at position 1191 (Fig. 1). A potential ribosome-binding site (AGGG) was identified just 6 bp upstream of the putative start codon. The deduced polypeptide product was 327 amino acids in length and showed 80.0% identity and 89.2% similarity to the deduced amino acid sequence of the UDP-glucose 4-epimerase of *E. coli* (Lemaire and Müller-Hill 1986).

An *exoB* disruption mutant of *R. leguminosarum* bv trifolii

Four Tn5 insertion mutants of plasmid pBSB43 were analyzed (Fig. 1). Two of them retained the ability to complement *R. meliloti* 7094 to form fluorescent colonies on Calcofluor LB agar and to nodulate alfalfa, but the other two had lost both phenotypes. Chromosomal *exoB* Tn5 insertions could not be obtained by following the plasmid incompatibility method (Ditta 1986). A disruption mutant of the chromosomal *exoB* gene was obtained by using gene *sacB* (Quandt and Hynes 1993), which makes cells unable to grow in the presence of sucrose. The Tn5 sequence in plasmid pBSB43-4 was excised by *Hpa*I digestion and replaced by a 2-kb *Sma*I fragment of plasmid pUTminiTn5 Sp/Sm that contains a spectinomycin resistance gene. The 5.7-kb *Pst*I fragment of the modified plasmid was subcloned into the *Pst*I site of plasmid pJQ200SK that contains gene *sacB*. The resulting plasmid, pJQ2003, was transferred from *E. coli* S17-1 to *R. leguminosarum* bv trifolii RS800 by selecting for resistance to spectinomycin and sucrose. The resulting colonies had undergone double recombination events that were confirmed by hybridization. The result was the replacement of the chromosomal *exoB* gene by the disrupted homolog from the plasmid. One of these strains was designated RS12.

Mutant RS12 grew more slowly than its parental strain, tended to flocculate, and formed deep red colonies on Congo red plates, whereas wild-type colonies were pale pink. A lack of EPS seems to increase the uptake of Congo red in other *exo* mutants (Breedveld et al. 1993).

The LPS profile of the wild-type strain (Fig. 2, lane 4) consisted of two major LPS species, a slow-migrating ladder-like structure (LPSII) which could represent the *O*-antigen-containing LPS, and the faster migrating band (LPSI) that consists of lipid A and core only (Carlson 1984). Our *exoB* mutant lacked the higher molecular weight form of the LPS, and contained only LPSII, which had a somewhat higher electrophoretic mobility than LPSII of wild type (Fig. 2, lane 5). The

![Image 318x104 to 522x257](Image 318x104 to 522x257)

![Image 319x262 to 523x272](Image 319x262 to 523x272)

**Fig. 1** Restriction map of the 3.7-kb *Pst*I fragment of the RS800 chromosomal DNA. *Bars* with open circles indicate Tn5 insertions that give rise to fluorescent colonies on Calcofluor media; insertions indicated by closed circles do not result in fluorescence. The numbers are the last digit in the name of the plasmid that bears the insertion (Table 1). The box indicates the *exoB* open reading frame and the arrow the direction of transcription. (*P*, *Pst*I, *S*, *Ssp*I, *Pv* *Pvu*I, *B* *Bam*HI, *R* *Rsa*I, *Sp* *Spa*I, *EV* EcoRV, *A* *Avu*I, *Sal*I)

![Image 43x121 to 286x202](Image 43x121 to 286x202)

**Fig. 2** SDS-polyacrylamide gel electrophoresis of lipopolysaccharides (LPS) from the wild-type RS800 (lane 4), the *exoB* mutant strain, RS12 (lane 5), and the same mutant with plasmid pBSB43 (lane 6). LPS from smooth *Salmonella typhimurium* (lane 1), and rough *S. typhimurium* TV119 (lane 2), and *S. typhimurium* SL1181 (lane 3) were used as standards
LPS profile of RS12 was restored to the wild-type LPS profile by complementation with plasmid pBSB43 that contains the complete exoB gene of *R. leguminosarum* bv *trifolii* RS800 (Fig. 2, lane 6).

Analysis of RS800 and RS12 EPS

The $^1$H-NMR spectrum of the EPS from the parent strain RS800 (Fig. 3A) was consistent with a polysaccharide bearing several different non-carbohydrate substitutions. The proton resonances between $\delta$ 3.0–5.0 were assigned to glycosyl components, and the resonances at $\delta$ 1.2, 1.4, and 2.1 were assigned to methyl protons of 3-hydroxybutyryl, pyruvate, and acetate group substitutions, respectively. GLC/MS analyses indicated that the EPS from this wild-type strain was an acidic heteropolysaccharide containing glucose, glucuronic acid, and galactose residues in an approximate glycosyl molar ratio of 4.5 : 1.2 : 1 (Fig. 4A). These $^1$H-NMR and GLC/MS data were similar to the Type 2 EPS previously shown to be made by *R. leguminosarum* bv *trifolii* wild-type strains ANU843, NA-30, and TA-1 (Hollingsworth et al. 1984; Philip-Hollingsworth et al. 1989). The oligosaccharide repeat unit of the Type 2 acidic EPS structure consists of an octasaccharide with two glucuronic acid residues and two glucose residues that constitute the tetrasaccharide main chain and a tetrasaccharide branch that contains three internal glucose residues and a galactose residue at the non-reducing terminus (Fig. 5). The multiplicity and splitting pattern of the non-carbohydrate resonances in the $^1$H-NMR spectrum of the EPS from strain RS800 (Fig. 3A) were consistent with their location in the Type 2 structure presented in Fig. 5. The single acetate group was primarily attached to O-3 (with minor migration to O-2) of the glucuronic acid residue adjacent to glucose in the main chain. The two pyruvate groups were attached to the 4 and 6 positions of the terminal galactose and adjacent glucose residues of the side chain, while the 3-hydroxybutyryl group was esterified on the O-3 position of the galactose residue.

From its $^1$H-NMR spectrum, the EPS from the *exoB* mutant (Fig. 3B) was similar to that of the RS800 parent, but lacked the 3-hydroxybutyrate moiety and one of the pyruvate groups ($\delta$ 1.2, 1.4). The other pyruvate group and the acetate group were still present in the *exoB* mutant EPS. GLC/MS analysis of the EPS from the *exoB* mutant (Fig. 4B) indicated the presence of glucose and glucuronic acid, in a ratio of 4.8 : 1.3, and a total absence of galactose.

Nodulation of clover by RS800 and RS12

Nodules induced by the RS800 wild-type strain were typical of an effective symbiosis. They were pink, cylindrical, normal in size (3–4 mm in *T. repens*, 5–6 mm in *T. subterraneum*), and developed mainly on primary roots. In contrast, nodules induced by the RS12 mutant strain were indicative of an ineffective symbiosis. They lacked the cylindrical shape and they were white, smaller, and more numerous per plant than those of the wild type, and frequently located at the site of secondary root emergence. The distribution of nodule development on roots was affected by the mutation of the bacterial exoB gene.

In contrast to the nodules induced by the RS800 wild-type strain in *T. subterraneum* (Fig. 6A,B), those induced by RS12 mutant strain were uninvaded and lacked infection threads and bacteroids. Host cells in the central region of these nodules were smaller and contained more starch grains than did host cells in nodules induced by wild-type RS800. Few nodules on plants inoculated with the RS12 mutant strain contained bacterial cells. In these rare cases, host cells that contained bacteria were located near the periphery of the central region (Fig. 6C,D). Deeper sections of the same nodules (Fig. 6E,F) revealed only uninfected host cells. These results indicated a significant restriction in the ability of the *exoB* mutant to invade host cells within clover nodules.

Transmission electron microscopy shows club-shaped bacteroids of wild-type RS800 enclosed within symbiosomes of infected nodule cells (Fig. 7A). Most host cells in the nodule central region were uninfected by the *exoB* mutant, RS12, and contained an abundance of starch granules (Fig. 7B). In the rare host cells that are invaded...
by the bacterial mutant, bacteria were released from infection threads but were not maintained in symbiosomes (Fig. 7C,D). Instead, the released bacteria were in direct contact with host cytosol, which itself appeared to undergo degradation and to accumulate numerous membraneous vesicles. The degeneration of the bacterial and host cell cytosol and the lack of intact peribacteroid membranes indicated an impaired root-nodule symbiosis.

Discussion

The present work is the first study of the exoB gene of R. leguminosarum bv trifolii, involved in polysaccharide synthesis. This gene is chromosomally located, and the original cosmid that contained the gene complemented none of the R. meliloti exoA, exoF or exoM mutants. In R. meliloti 1021, exoB maps in a cluster of exo genes that includes exoA, exoF, and exoM, on the second symbiotic megaplasmid (Leigh and Walker 1994). Therefore, the genomic distribution of the exo loci differs in these two Rhizobium species. As in R. leguminosarum by trifolii, exoB maps on the chromosome of other Rhizobiaceae species including one that nodulates H. coronarium (Ollero et al. 1994), and one that nodulates Acacia (Lopez-Lara et al. 1995) and Agrobacterium tumefaciens (Cangelosi et al. 1987). Despite the high divergence in genomic organization of the exo loci between R. meliloti and R. leguminosarum by trifolii, DNA sequence similarity and functional interchangeability indicated parallel evolution of the exoB genes in both species.

The EPS produced by R. leguminosarum by trifolii wild-type strain corresponded to the Type 2 EPS synthesized by one of the major groups of R. leguminosarum by trifolii strains. This EPS bears a single acetyl group on O-3 of one of the glucuronic acid residues in the backbone and contains only half as much 3-hydroxybutyryl groups as R. leguminosarum bv viciae (Philip-Hollingsworth et al. 1989). The 1H-NMR and GLC/MS analyses of the acidic EPS produced by RS800 and RS12 exoB mutant strains were fully consistent with the assumed function of ExoB. Since the exoB mutant

Fig. 4 Total ion chromatogram of alditol acetate derivatives of glycosyl components from exopolysaccharide of wild type, RS800 (A) and the exoB mutant, RS12 (B). Peak 2 is the galactosyl derivative, whereas peak 3 contains both the glucosyl and glucuronosyl derivatives, respectively. The latter two components are distinguished by selected ion monitoring mass spectrometry. Peak 1 represents a trace of the decarboxylated product of uronic acid.

Fig. 5 Structure of R. leguminosarum by trifolii RS800 exopolysaccharide (EPS). Diagram showing the octasaccharide repeated unit of RS800 EPS. (Glc Glucose, Gal galactose, GlcA glucuronic acid, Pyr pyruvyl group, OAc acetyl group, 3HB 3-hydroxybutyryl group)

Fig. 6 Light micrographs of longitudinal nodule sections of Trifolium subterraneum inoculated with R. leguminosarum by trifolii wild type, RS800 (A, B) or the exoB mutant, RS12 (C–F). B, D, and F are higher magnifications of the center of nodule sections in A, C, and E, respectively. (Bar scales 20 μm (B, D, F) and 100 μm (A, C, E)}
cannot make the precursor for galactose incorporation, the EPS repeat unit that it makes lacks the terminal galactose in the branch, thus eliminating the site for attachment of the 3-hydroxybutyrate substitution and one of the pyruvate substitutions. This explains how mutation of a gene responsible for interconversion of a glycosyl component of EPS affects not only the incorporation of that carbohydrate residue but also of non-carbohydrate substitutions attached to it in the synthesis of the EPS repeat unit structure.

Although a chemical analysis of LPS in the \( \text{exoB} \) mutant was not performed, a possible model to account for its altered electrophoretic profile is that the \( \text{exoB} \) mutation blocked the synthesis of the galactose-containing tetrasaccharide core (Hollingsworth et al. 1989) to which the \( O \)-antigen is normally attached. Because ExoB is required for the biosynthesis of precursors of EPS and LPS, a pleiotropic effect on both polymers has also been described for \( \text{exoB} \) mutants of \( R. \text{leguminosarum bv vicieae} \) (Canter Cremers et al. 1990), a \( Rhizobium \) species that nodulates \( H. \text{coronarium} \) (Ollero et al. 1994), and one that nodulates \( Acacia \) (Lopez-Lara et al. 1995). Since EPS and LPS of RS12 were affected by the \( \text{exoB} \) mutation, the symbiotic defect of this mutant could not be attributed to alterations in any one of these two surface glycoconjugates.

\( R. \text{leguminosarum bv vicieae} \) \( \text{exoB} \) mutants fail to induce nodules or nodule-like structures on host plants (Canter Cremers et al. 1990). In contrast, the \( R. \text{leguminosarum bv trifolii} \) \( \text{exoB} \) mutant induced nodule

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**Fig. 7** Transmission electron micrographs of nodule sections of \( T. \text{subterraneum} \) inoculated with \( R. \text{leguminosarum bv trifolii} \) wild type, RS800 (A), or the \( \text{exoB} \) mutant, RS12 (B–D). \textit{Bar scales} are 1 \( \mu \text{m} \) in A, C, and D; 2 \( \mu \text{m} \) in B.
formation of clovers, but their differentiation and distribution on the root differed considerably from nodules induced by the wild-type parent. Thus, the symbiotic phenotypes of $exoB$ mutants of these closely related rhizobia are not identical. This implies that the requirement(s) for galactosyl-containing glycoconjugates of rhizobia may be more stringent in certain symbioses than others. Our results are consistent with the data of Derylo et al. (1986) which showed that exopolysaccharide deficient mutants derived from $R.\ leguminosarum$ bv $trifolii$ 24AR5 were able to induce early steps of nodulation, although only a small number of bacteria were released from infection threads and some of them appeared to be lysed. Moreover, our results on the majority of RS12-induced nodules are in accordance with the analysis of $R.\ meliloti$ 1021 $exo$ mutant-induced nodules (Yang et al. 1992). These nodules also develop more frequently on secondary roots than on the primary root. The $exo$ mutant-induced infection threads abort within the peripheral cells, and the nodules lack a well-defined apical meristem.

In summary, our studies show that mutation of the $exoB$ gene in $R.\ leguminosarum$ bv $trifolii$ leads to important alterations in EPS and LPS structures, and to symbiotic defects in nodule development, invasion of nodule host cells, and sustained maintenance of an intracellular endosymbiotic state.

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