The *Sinorhizobium meliloti* ExpE1 protein secreted by a type I secretion system involving ExpD1 and ExpD2 is required for biosynthesis or secretion of the exopolysaccharide galactoglucan

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In *Sinorhizobium meliloti* the biosynthesis of the exopolysaccharide galactoglucan (EPS II) is directed by the *exp* genes. The *expD1* and *expD2* gene products are homologous to components of type I secretion systems. ExpE1, the gene of which is located adjacent to *expD1* and *expD2*, was detected in *S. meliloti* cells and culture supernatants. ExpD1 and ExpD2 were required for the secretion of ExpE1, indicating that ExpE1 is secreted by a type I secretion system involving ExpD1 and ExpD2. ExpE1 contains 15 aspartate- and glycine-rich nonapeptide repeats that were suggested to bind Ca²⁺. The ability to bind Ca²⁺ was demonstrated for a recombinant ExpE1 protein. Extracellular EPS II was not detected in cultures of non-polar *expD1*, *expD2* and *expE1* deletion mutants implying that these three genes are required for biosynthesis or secretion of galactoglucan.

Keywords: *Rhizobium meliloti*, exopolysaccharides, type I secretion, Ca²⁺-binding protein

INTRODUCTION

Bacterial exopolysaccharides (EPSs) are essential for the establishment of the nitrogen-fixing symbiosis between *Sinorhizobium meliloti* and the host plant *Medicago sativa* (Leigh & Walker, 1994). Bacterial mutants unable to produce EPS induce the formation of root nodules on their host plant but fail to invade these nodules (González *et al.*, 1996; Leigh & Walker, 1994). Under standard conditions, *S. meliloti* wild-type strain SU47 and its streptomycin-resistant derivative strains 1021 and 2011 secrete an acidic EPS termed succinoglycan (EPS I). This EPS is composed of octasaccharide repeating units containing one galactose and seven glucose residues, and can be decorated by acetyl, succinyl and pyruvyl groups (Reinhold *et al.*, 1994; Wang *et al.*, 1999). The *exo/exs* gene cluster responsible

for the biosynthesis of EPS I has been extensively studied and functions were proposed for most of the *exo* and *exs* gene products (Becker & Pühler, 1998; Leigh & Walker, 1994).

In addition, *S. meliloti* strains 1021 and 2011 have the ability to produce a second EPS, termed galactoglucan (EPS II), which can replace EPS I in nodule invasion (Glazebrook & Walker, 1989; González *et al.*, 1996; Zhan *et al.*, 1989). EPS II is composed of alternating glucose and galactose residues which are decorated by acetyl and pyruvyl groups (Glazebrook & Walker, 1989). Under standard culture conditions in complex media, large amounts of EPS I and almost no EPS II are produced. The production of EPS II was observed in the presence of a mutation in either of the regulatory genes *expR* (Glazebrook & Walker, 1989) or *mucR* (Keller *et al.*, 1995; Zhan *et al.*, 1989). In addition, Zhan *et al.* (1991) showed that under phosphate-limiting conditions the wild-type strain 1021 produces EPS II. Extra copies of the *exp* gene cluster responsible for EPS II biosynthesis or extra copies of the transcriptional regulator *expG* also resulted in the biosynthesis of EPS II (Glazebrook & Walker, 1989; Rüberg *et al.*, 1999; Zhan *et al.*, 1989).

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Abbreviations: ABC, ATP-binding cassette; EPS, exopolysaccharide; EPS I, succinoglycan; EPS II, galactoglucan; HMM, high molecular mass; LMM, low molecular mass; MBP, maltose-binding protein; MCS, multiple cloning site.

The sequence of the *exp* gene cluster directing the biosynthesis of EPS II was previously reported and the inferred properties of the encoded gene products implied that these proteins are involved in regulation of *exp* gene expression, diphospho-nucleotide sugar biosynthesis, polymerization and export (Becker *et al.*, 1997). Homologies of the deduced amino acid sequences of ExpD1 and ExpD2 to components of type I secretion systems implied that the N-terminal domain of ExpD1 represents a membrane-spanning domain and the C-terminal portion is the ATP-binding cassette (ABC) domain (Becker *et al.*, 1997). ExpD2 displays homologies to accessory factors of these secretion systems designated membrane fusion proteins (MFPs). Secretion complexes consisting of an ABC transporter and a MFP in conjunction with an outer-membrane protein are involved in the signal-peptide-independent secretion of proteins which usually contain a C-terminal secretion signal (Dinh *et al.*, 1994). Most of these secreted proteins contain several aspartate- and glycine-rich nonapeptide tandem repeats involved in the binding of Ca^{2+} near the C-terminal secretion signal (Baumann, 1994; Economou *et al.*, 1990; Ludwig *et al.*, 1988).

Genes encoding type I secretion systems are usually situated adjacent to the genes encoding the secreted proteins (Fath & Kolter, 1993). The protein encoded by *expE1*, the gene for which is located immediately downstream of the *expD1-expD2* operon, contains 15 aspartate- and glycine-rich nonapeptide repeats, and the structural characteristics of the C-terminal portion of ExpE1 are similar to those of C-terminal secretion signals (Becker *et al.*, 1997). These features of ExpE1 suggest that this protein is a candidate to be secreted by a transporter complex involving ExpD1 and ExpD2. In this study we have analysed the role of *expD1* and *expD2* in secretion of ExpE1 and the involvement of the *expD1*, *expD2* and *expE1* genes in EPS II production.

METHODS

Microbiological methods. Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in superbroth (Ausubel *et al.*, 1990) or in LB medium (Sambrook *et al.*, 1989) at 37 °C. *S. meliloti* strains were grown in TY (Beringer, 1974), LB or MOPS-buffered medium supplemented with 0.1 mM phosphate (Neidhardt *et al.*, 1974) at 30 °C. Antibiotics were supplemented as required at the following concentrations ($\mu\text{g ml}^{-1}$): for *S. meliloti*, spectinomycin, 200; streptomycin, 600; nalidixic acid, 8; tetracycline, 8; gentamicin, 40; and neomycin, 120; for *E. coli*, tetracycline, 10; ampicillin, 100; gentamicin, 10; chloramphenicol, 50; and kanamycin, 50.

DNA manipulations. Preparation of plasmid DNA, DNA restriction, agarose gel electrophoresis, cloning and transformation of *E. coli* cells were carried out according to established protocols (Sambrook *et al.*, 1989). DNA was sequenced with the ΔTaq cycle sequencing kit (Amersham), using Cy5-labelled primers and processed using the ALF DNA Express Sequencer (Pharmacia) according to the manufacturer's instructions. Southern hybridizations were performed as described by Kessler (1992). Total DNA from rhizobia was isolated as described by Meade *et al.* (1982).

Mutations were transferred between *S. meliloti* strains by phage ΦM12 -mediated general transduction as described by Finan *et al.* (1984). Transfer of the mutations was verified by Southern hybridizations.

Construction of *expD1*, *expD2* and *expE1* deletion mutants.

The 7633 bp *KpnI-EcoRI* fragment of the *exp* gene cluster of *S. meliloti* 2011 (Becker *et al.*, 1997) containing a deletion that ranged from nt 36 to nt 1756 of the 1770 nt *expD1* coding region (*expD1* _{$\Delta 36-1756$}) was cloned into the multiple cloning site (MCS) of suicide vector pK18mobG (Katzen *et al.*, 1999). Start and stop codons of the remaining *expD1* coding region were in-frame due to the presence of some nucleotides from the pK18mobG MCS. The same procedure was followed for the deletion of large parts of the *expD2* and *expE1* genes. The 4182 bp *BglII-EcoRI* fragment and the 6292 bp *BalI-HindIII* fragment of the *exp* gene cluster containing respectively, a deletion ranging from nt 37 to nt 1009 of the 1422 nt *expD2* coding region (*expD2* _{$\Delta 37-1009$}) and a deletion ranging from nt 2 to nt 616 of the 660 nt *expE1* coding region (*expE1* _{$\Delta 2-616$}) were inserted into the MCS of pK19mobG or pK18mobG. The plasmids obtained were designated pLM82-4, pLM87-1 and pLM811-3, characterized by deletions in *expD1*, *expD2* and *expE1*, respectively. These plasmids were mobilized from strain *E. coli* S17-1 (Simon *et al.*, 1983) to *S. meliloti* 2011. Homogenization of these mutations was carried out as described by Katzen *et al.* (1999).

Plasmids pLM91-1 and pLM93-2 were obtained by insertion of a 2268 bp *PvuII-EcoRI* fragment containing *expE1* under the control of its own promoter into the broad-host-range vector pSUP104 (Priefer *et al.*, 1985) and suicide vector pK18mob (Schäfer *et al.*, 1994), respectively. These plasmids were transferred to RmLM9835 and RmLM9836. Plasmid pLM811-1 was obtained by cloning a 3927 bp *BalI* fragment containing *expD1* and *expD2* under the control of the promoter of the *expD* operon into vector pK18mobG (Katzen *et al.*, 1999). This plasmid was mobilized to SmLM9828 and SmLM9832.

To obtain plasmid pExpE7-*lacZ* a promoterless *lacZ*-Gm interposon (Becker *et al.*, 1995) was inserted in sense orientation into the *EcoRV* site of *expE7* cloned into pK18mob. This plasmid was homogenized to Rm101 (Becker *et al.*, 1997) and SmLM9909. These strains were grown until OD₆₀₀ 0.5–0.6 in LB medium and assayed for β -galactosidase activity as described by Miller (1972).

All plasmid integrations into the genome and homogenizations were verified by Southern hybridizations.

Purification of recombinant ExpE1. The 980 bp *BalI* fragment from pAB56-9 (Table 1) containing *expE1* was inserted into the *BamHI* site of pWH844 (Schirmer *et al.*, 1997) blunted by S1 nuclease. Correct fusion between the *expE1* structural gene and the (His)₆-encoding region of pWH844 was verified by DNA sequencing. The resulting plasmid was designated pLM75-2 and transferred to *E. coli* SURE (Stratagene).

Purification of ExpE1 was performed using the QIA-expressionist protein purification system (Qiagen) with some modifications. After lysis, the cell-free extract was loaded onto a nickel-nitrilotriacetic acid (Ni-NTA) agarose column (Superflow) equilibrated in lysis buffer. The column was washed with 6 vols buffer A (50 mM Tris/HCl, 300 mM NaCl, 10%, v/v, glycerol, pH 7.5) and 6 vols buffer B (100 mM Tris/HCl, 500 mM NaCl, pH 8.9). Bound proteins were eluted with buffer B by using a step gradient containing 0–0.5 M imidazole. Proteins from collected fractions were visualized by vertical SDS-PAGE with 15% acrylamide (Laemmli, 1970) stained with Coomassie brilliant blue R250.

Table 1. Strains, plasmids and phage

Nx, nalidixic acid; Sm, streptomycin; Spc, spectinomycin; Tc, tetracycline; Km, kanamycin; Ap, ampicillin; Cm, chloramphenicol, Gm, gentamycin.

Strain/plasmid/ phage	Relevant characteristics	Source/reference
Strains		
<i>S. meliloti</i>		
Rm2011	Wild-type, Nx ^r Sm ^r	Casse <i>et al.</i> (1979)
Rm101	Rm2011, Spc ^r cassette of pHP45Ω inserted into the <i>Pmacl</i> site of <i>mucR</i> (<i>mucR101</i> – <i>spc</i>)	Becker <i>et al.</i> (1997)
RmAR9007	Rm2011, <i>exoY</i> – <i>lacZ</i> / <i>aacC1</i>	Keller <i>et al.</i> (1995)
RmAR9007 TD101	Rm2011, <i>exoY</i> – <i>lacZ</i> / <i>aacC1 mucR</i> – <i>spc</i>	I. Quester*
SmLM8311	RmAR9007 TD101, <i>expA3</i> ::Tn5	This work
RmAR1157	Rm2011, <i>exoY</i> ::Tn5 <i>expA1</i> – <i>lacZ</i> / <i>aacC1</i>	A. Roxlau*
SmLM9806	Rm2011, <i>ΔexpD1</i>	This work
SmLM9829	Rm2011, <i>ΔexpD2</i>	This work
SmLM9833	Rm2011, <i>ΔexpE1</i>	This work
SmLM9815	Rm2011, <i>ΔexpD1 exoY</i> – <i>lacZ</i> / <i>aacC1</i>	This work
SmLM9831	Rm2011, <i>ΔexpD2 exoY</i> – <i>lacZ</i> / <i>aacC1</i>	This work
SmLM9835	Rm2011, <i>ΔexpE1 exoY</i> – <i>lacZ</i> / <i>aacC1</i>	This work
SmLM9909	Rm2011, <i>ΔexpE1 mucR</i> – <i>spc</i>	This work
SmLM9901	SmLM9835 carrying pLM91-1	This work
SmLM9903	SmLM9835 carrying pLM93-2 integrated into the genome	This work
SmLM9906	SmLM9815 carrying pLM91-1	This work
SmLM9828	Rm2011, <i>ΔexpD1 exoY</i> – <i>lacZ</i> / <i>AaccC1 mucR</i> – <i>spc</i>	This work
SmLM9832	Rm2011, <i>ΔexpD2 exoY</i> – <i>lacZ</i> / <i>aacC1 mucR</i> – <i>spc</i>	This work
SmLM9836	Rm2011, <i>ΔexpE1 exoY</i> – <i>lacZ</i> / <i>aacC1 mucR</i> – <i>spc</i>	This work
SmLM9904	SmLM9836 with pLM93-2 integrated into the genome upstream of the <i>expE1</i> deletion	This work
SmLM9905	SmLM9836 with pLM93-2 integrated into the genome downstream of the <i>expE1</i> deletion	This work
SmLM9907	SmLM9828 carrying plasmid pLM811-1 integrated into the genome	This work
SmLM9908	SmLM9832 carrying plasmid pLM811-1 integrated into the genome	This work
<i>E. coli</i>		
XL1-Blue S17-1	<i>recA1 lac</i> [F' <i>proAB lacI</i> ^a ZΔM15 Tn10 (Tc ^r)] <i>thi</i> <i>E. coli</i> 294, <i>thi</i> RP4-2–Tc::Mu–Km::Tn7 chromosomally integrated	Bullock <i>et al.</i> (1987) Simon <i>et al.</i> (1983)
DH5α	<i>recA1, lacUi69, O80dlacZ ΔM15</i>	BRL
SURE	<i>e14</i> [–] (<i>mcrA</i>) Δ(<i>mcrCB</i> – <i>hsdSMR</i> – <i>mrr</i>)171 <i>endA1 supE44 thi-1 gyrA96 relA1 lac recB recJ sbsC umuC</i> ::Tn5 (Km ^r) <i>uvrC</i> [F' <i>proAB, lacI</i> ^a ZΔM15 Tn10 (Tc ^r)]	Stratagene
Plasmids		
pAB56-9	pUK21 containing a 4.1 kb <i>KpnI</i> – <i>HindIII</i> fragment of the <i>exp</i> gene cluster	This work
pWH844	pQE9 derivative, <i>lacI</i> ^a , Ap ^r	Schirmer <i>et al.</i> (1997)
pMAL-p	<i>malE</i> –(MCS)– <i>lacZα</i> fusion, <i>lacI</i> ^a , Ap ^r	New England Biolabs
pK18/19mob	pUC18/19 derivative, <i>lacZα, mob</i> , Km ^r	Schäfer <i>et al.</i> (1994)
pK18/19mobG	pK18/19mob derivative, <i>gusA</i>	Katzen <i>et al.</i> (1999)
pSUP104	Broad-host-range plasmid pACYC184 derivative, <i>mob</i> , Tc ^r , Cm ^r	Priefer <i>et al.</i> (1985)

Table 1 (cont.)

Strain/plasmid/ phage	Relevant characteristics	Source/reference
pLM82-4	pK18mobG containing a 7633 bp <i>KpnI</i> – <i>EcoRI</i> fragment from <i>exp</i> region with a 1720 nt <i>expD1</i> deletion	This work
pLM87-1	pK19mobG containing a 4182 bp <i>BglII</i> – <i>EcoRI</i> fragment from <i>exp</i> region with a 972 nt <i>expD2</i> deletion	This work
PLM811-3	pK18mobG containing a 6292 bp <i>BalI</i> – <i>HindIII</i> fragment from <i>exp</i> region with a 614 nt <i>expE1</i> deletion	This work
pLM91-1	pSUP104 containing a 2268 bp <i>PvuII</i> – <i>EcoRI</i> fragment with the <i>expE1</i> gene under the control of its own promoter	This work
pLM93-2	pK18mob containing a 2268 bp <i>PvuII</i> – <i>EcoRI</i> fragment with <i>expE1</i> gene under the control of its own promoter	This work
pLM811-1	pK18mobG containing a 3927 bp <i>BalI</i> fragment with <i>expD1</i> and <i>expD2</i> genes under the control of the <i>expD</i> operon promoter	This work
pLM76-4	258 nt of the 3' terminus of the <i>expE1</i> coding region cloned into the <i>StuI</i> site of the pMAL-p expression vector	This work
pLM76-2	pMAL-p containing the complete <i>expE1</i> gene cloned in the <i>StuI</i> site	This work
pLM75-2	pWH844 containing the <i>BalI</i> fragment encompassing the <i>expE1</i> gene	This work
pLM83-1	pWH844 derivative containing the complete <i>expE1</i> gene	This work
pLM82-6	pK19mob expressing a translational fusion ExpE1–LacZ	This work
pExpE7–lacZ	Promoterless <i>lacZ</i> –Gm interposon inserted into the <i>EcoRV</i> site of the <i>expE7</i> coding region	Becker <i>et al.</i> (1997)
Phage ΦM12	<i>S. meliloti</i> transducing phage	Finan <i>et al.</i> (1984)

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The most purified fractions of (His)₆ExpE1 were pooled, concentrated in an Ultrafree Concentrator (molecular mass cutoff, 10 kDa; Millipore) and then separated on a Sephacryl S200 HR column (16 mm diameter, 600 mm length) according to the manufacturer's recommendations (Pharmacia) with buffer B as the mobile phase. The eluted (His)₆ExpE1 was associated with a major protein peak as confirmed by SDS-PAGE. The purification of (His)₆ExpE1 to electrophoretic homogeneity was achieved by preparative gel electrophoresis (model 491 Prep Cell; Bio-Rad) according to the manufacturer's instructions. N-terminal protein sequencing was performed by the Max-Planck-Institut für Molekulare Physiologie.

Affinity purification of anti-ExpE1 antibodies. The pure ExpE1 containing the (His)₆ tag was used for immunization of rabbits (Eurogentec). The serum obtained was subjected to immunoaffinity purification using the 85 C-terminal amino acids from ExpE1 fused in-frame to the maltose-binding

protein (MBP). To obtain this fusion protein, the last 258 nt of *expE1* were amplified by PCR (upper primer 5'-GGCAGC-GATATCTTCGTT-3'; lower primer 5'-CTATCAGTGGA-CAGTGAAGTAGTT-3') and cloned into the *StuI* site of vector pMAL-p (New England Biolabs) resulting in pLM76-4. Purification of MBP–ExpE1_{C-term} was performed according to a standard protocol (New England Biolabs) with some minor modifications. The osmotic-shock fluid containing the fusion protein was loaded onto an amylose resin column and the buffers used in the washing steps did not include Tween 20. The eluted protein was applied to a SDS-PAGE and electroblotted onto a nitrocellulose membrane. After protein staining with Ponceau S, the band corresponding to the MBP–ExpE1_{C-term} fusion was removed and used as an immobilized antigen. Antibody affinity purification was performed as described by Harlow & Lane (1988).

⁴⁵Ca²⁺ binding. To test the calcium-binding activity of ExpE1,

the corresponding gene was amplified by PCR (upper primer 5'-ATGGCCACTTTGGAAGG-3'; lower primer 5'-CTATC-AGTGGACAGTGAAGTAGTT-3') and cloned in-frame with the MBP gene from pMAL-p, producing pLM76-2. This fusion protein (MBP-ExpE1) was purified by affinity chromatography as described above. MBP was cleaved from ExpE1 by protease factor Xa (2 d at 4 °C) resulting in the removal of vector-derived residues attached to ExpE1. The protein mixture containing ExpE1 was subjected to SDS-PAGE, transferred onto a nitrocellulose membrane and treated as described by Maruyama *et al.* (1984) to identify Ca²⁺-binding proteins.

Immunodetection of proteins. Proteins separated by SDS-PAGE were electroblotted onto nitrocellulose membranes by the procedure of Towbin *et al.* (1979). Immunodetection was performed according to standard protocols (Harlow & Lane, 1988).

Analysis of ExpE1 in cell extract and culture supernatant. *S. meliloti* strains in the late-exponential growth phase (OD₆₀₀ 1.5–2.0) were grown in MOPS-buffered medium supplemented with 0.1 mM phosphate. Cells were centrifuged for 5 min at 18 000 g at 4 °C and the pellets were solubilized in SDS sample buffer to yield a preparation of total cellular proteins. Culture supernatants were separated from the cells by centrifugation (20 000 g, 4 °C, 30 min) and a protease-inhibitor cocktail (EDTA free; Boehringer) was added. A volume of 8 ml of each supernatant was concentrated by filtration (Ultrafree Concentrator) and mixed with SDS sample buffer. Preparation of outer-membrane proteins from *S. meliloti* was performed according to a protocol described by Poxton *et al.* (1985). Protein concentrations were determined by using the Bio-Rad Protein Assay with BSA as a standard.

Analysis of extracellular carbohydrates in culture supernatants. *S. meliloti* strains were grown at 30 °C for 10 d in MOPS-buffered medium supplemented with 0.1 mM phosphate. Cells were removed by centrifugation (20 000 g, 1 h) and the culture supernatants were desalted by dialysis (molecular mass cutoff 1 kDa) against water for 4 d, followed by EPS concentration by lyophilization. High- (HMM) and low-molecular-mass (LMM) fractions were separated by gel-filtration chromatography (twice on GFC 4000-8 and once on GFC 300-8, column size 300 × 7.7 mm; Machery-Nagel; flow rate, 0.8 ml min⁻¹; 200 mM sodium chloride, 200 mM sodium phosphate buffer, pH 7.0). Fractions were detected using a differential refraction index detector. Total carbohydrates were quantified by the HCl/L-cysteine method (Chaplin & Kennedy, 1986). Glucose and galactose content of the HMM and LMM fractions was quantified by enzymic assays after hydrolysis with hydrochloric acid and subsequent neutralization (Beutler, 1984; Kunst *et al.*, 1984).

RESULTS

An antiserum directed against the C-terminal portion of ExpE1 comprising 85 amino acids is specific for the ExpE1 protein

A (His)₆ExpE1 fusion protein was expressed from pLM75-2 in *E. coli* SURE (Fig. 1a, lane 1). SDS-PAGE of the protein purified by affinity chromatography exhibited a major band migrating at approximately 34 kDa (Fig. 1a, lane 2), which reacted with anti-His-tag antibody (data not shown), but whose mass did not correspond to the calculated molecular mass of the N-

Fig. 1. (a) Purification of (His)₆ExpE1. Coomassie blue-stained SDS-PAGE gel displaying the molecular mass marker Dalton VII-L (M); cell extract of *E. coli* SURE producing (His)₆ExpE1 (from pLM75-2) grown in the presence of 1 mM IPTG (lane 1); sample of the peak fraction eluted from Ni-NTA affinity chromatography (lane 2); sample of the peak fraction after gel filtration (lane 3); purified (His)₆ExpE1 (3 µg protein) after preparative gel electrophoresis separation (lane 4). (b) Western blot analysis of ExpE1 in total-cell proteins of *S. meliloti* SmLM8311 (lane 1), *E. coli* SURE producing ExpE1 (pLM83-1) (lane 2) and the pure (His)₆ExpE1 (lane 3), using anti-ExpE1_{C-term} polyclonal antibodies.

terminal histidine-tagged ExpE1 (23 240 Da). Further purification was achieved by size-fractionation chromatography (Fig. 1a, lane 3). The *M_r* of the native (His)₆ExpE1 was estimated to be approximately 46 kDa (data not shown). Since the predicted *M_r* based on the DNA sequence is 23.2 kDa, it is likely that the native protein purifies as a dimer. The essentially pure (His)₆ExpE1 protein that was obtained by preparative gel electrophoresis migrated as a single 34 kDa band on a Coomassie blue-stained SDS-PAGE gel (Fig. 1a, lane 4). This protein was found to have an N-terminal 20 aa sequence that perfectly matched the predicted amino acid sequence of the engineered His-tagged ExpE1.

A polyclonal antiserum raised against the purified (His)₆ExpE1 detected the recombinant protein in total protein extracts from the *E. coli* expression strain. (His)₆ExpE1 could be detected with an antiserum dilution ranging from 1:1000 to 1:6000. Similar experiments were carried out in *S. meliloti* showing that this polyclonal antibody detected ExpE1 but also other proteins. No signal for the *S. meliloti* cell extract was detected with the preimmune serum. To overcome this lack of specificity, the polyclonal antibody was subjected to immunoaffinity purification using the 85 aa of the C-terminal part of ExpE1. This C-terminal region was chosen because ExpE1 contains 15 aspartate- and glycine-rich nonapeptide repeats in the 124 N-terminal aa and none in the last 85 aa (Becker *et al.*, 1997). Similar aspartate- and glycine-rich repeats, which might be recognized by the anti-(His)₆ExpE1 antiserum, were also identified in the ExsH and EglC proteins from *S. meliloti* (Sharypova *et al.*, 1999; York & Walker, 1997) and might also be present in additional *S. meliloti*

Fig. 2. Calcium-binding activity of ExpE1. ExpE1 protein obtained after cleavage of the MBP-ExpE1 fusion with factor Xa (lanes 3) or expressed by *E. coli* carrying pLM83-1 (lanes 4) was separated by SDS-PAGE and electroblotted. The protein fraction eluted from the amylose affinity chromatography containing MBP-ExpE1 expressed by pLM76-2 (lanes 1) and MBP expressed by pMAL-p (lanes 2) were included as controls. (a) Proteins stained with Ponceau S. (b) ExpE1 detection by autoradiography after incubation with $^{45}\text{Ca}^{2+}$. Lane M is marker Dalton VII-L.

proteins. Incubation of the purified immobilized MBP-ExpE1_{C-term} fusion protein with the polyclonal antibody anti-(His)₆ExpE1 resulted in the purified serum anti-ExpE1_{C-term} that reacted exclusively with the ExpE1 protein from *S. meliloti* cell extracts (Fig. 1b, lane 1), and detected ExpE1 in *E. coli* cell extracts as well as the purified (His)₆ExpE1 protein (Fig. 1b, lanes 2 and 3). Some additional signals were caused by degradation fragments of ExpE1, which migrated faster in the gel (Fig. 1b, lanes 1 and 2).

As described for (His)₆ExpE1, ExpE1 expressed in *E. coli* or *S. meliloti* also migrated abnormally in SDS-PAGE. ExpE1 has a predicted M_r 22 117, but it migrated at approximately 30 kDa (Fig. 1b, lanes 1 and 2).

ExpE1 binds Ca^{2+}

Since ExpE1 contains 15 aspartate- and glycine-rich nonapeptide repeats, the ability of ExpE1 to bind Ca^{2+} was assayed. The (His)₆ExpE1 protein could not be used for this assay due to the capacity of the histidine tag to bind divalent cations, therefore the *expE1* gene was expressed as a fusion with MBP in *E. coli* SURE. The *E. coli* cell extract was subjected to affinity chromatography and we observed, after SDS-PAGE, that beside the fusion protein MBP-ExpE1 at 64 kDa, another intense band running at approximately 42 kDa was present (Fig. 2a, lane 1) that possibly represented the MBP alone. Such an observation was also reported by Maina *et al.* (1988) and can be explained by premature termination or cleavage of the fusion protein. Incubation of this MBP-ExpE1 eluted fraction with the protease factor Xa resulted in the release of ExpE1. This

cleavage gave a 42 kDa band corresponding to the released MBP and a 30 kDa band corresponding to ExpE1 (Fig. 2a, lane 3). Due to premature termination or cleavage of the fusion protein and some degradation of ExpE1, the concentration of MBP was higher than that of ExpE1 (Fig. 2a, lane 3). Since the cleavage with factor Xa was not complete, a 64 kDa band corresponding to the non-cleaved MBP-ExpE1 was still present (Fig. 2a, lane 3). The eluted proteins together with protein cell extract from *E. coli* expressing the *expE1* gene were tested for binding of $^{45}\text{Ca}^{2+}$. A band migrating at approximately 64 kDa that corresponded to the fusion protein MBP-ExpE1 (Fig. 2b, lanes 1 and 3) and a band migrating at 30 kDa representing ExpE1 (Fig. 2b, lane 3) was detected due to binding of $^{45}\text{Ca}^{2+}$. In this assay, ExpE1 was also detected in the protein cell extract from *E. coli* expressing the native ExpE1 protein (Fig. 2b, lane 4) but no signal was obtained from *E. coli* cell extract lacking the *expE1* gene (data not shown). The observation that in Fig. 2b, lane 1, we obtain a radioactive signal only for the MBP-ExpE1 fusion but no signal with the cell extract in which MBP was expressed alone (Fig. 2b, lane 2), indicates that the binding of Ca^{2+} is due to ExpE1 and not to other *E. coli* proteins that may co-migrate with ExpE1.

ExpE1 is secreted by a transporter system involving ExpD1 and ExpD2

To investigate the involvement of the *expD1*, *expD2* and *expE1* genes in ExpE1 protein secretion and EPS II production, chromosomal mutants were generated using a gene-replacement strategy, whereby large parts of each of these genes were deleted giving rise to non-polar mutants. The deletion in *expD1* extended from nt 36 to nt 1756 and the remaining sequence that contained some nucleotides from the vector encoded a peptide of 31 aa. To mutate *expD2*, a fragment ranging from nt 37 to nt 1009 was deleted. The remaining sequence that included some nucleotides from the vector coded for a protein of 169 aa. The deletion from nt 2 to nt 616 of *expE1* resulted in a remaining sequence that contained some nucleotides from the vector and encoded a peptide of 22 aa.

Under standard conditions, Rm2011 produces EPS I and only traces of EPS II, if any. However, in a *mucR* or *expR* background and under phosphate-limiting conditions, synthesis of EPS II is stimulated (Glazebrook & Walker, 1989; Keller *et al.*, 1995; Zhan *et al.*, 1991). To overexpress the *exp* genes leading to the biosynthesis of EPS, the *mucR101-spc* mutation (Becker *et al.*, 1997) was transferred to each of the deletion mutants. To block biosynthesis of EPS I, a *lacZ-aacC1* insertion in the *exoY* gene (Keller *et al.*, 1995) encoding the galactose-1-phosphate transferase that catalyses the first step in the synthesis of the EPS I subunit (Reuber & Walker, 1993), was additionally transferred to the deletion mutants. The strains obtained were designated SmLM9815 (Rm2011 $\Delta\text{expD1}\text{exoY}$), SmLM9828 (Rm2011 $\Delta\text{expD1}\text{exoYmucR}$), SmLM9831 (Rm2011

Fig. 3. Western blot analysis of ExpE1 in total-cell proteins (a) and supernatants (b) of *S. meliloti* strains using anti-ExpE1_{C-term} polyclonal antibody. Lanes contain either the cells (without supernatant) from the equivalent of 10 µl culture (OD₆₀₀ 1.5–2.0) (a) or 2 ml cell-free concentrated supernatants (b). RmAR1157 (lanes 1), SmLM9815 (lanes 2), SmLM9831 (lanes 3), SmLM9835 (lanes 4), SmLM9901 (lanes 5), SmLM9903 (lanes 6) and SmLM9906 (lanes 7) are shown. Arrows indicate the position of the protein band corresponding to ExpE1.

ΔexpD2exoY), SmLM9832 (Rm2011 *ΔexpD2exoY-mucR*), SmLM9835 (Rm2011 *ΔexpE1exoY*) and SmLM9836 (Rm2011 *ΔexpE1exoYmucR*) (Table 1). Blocking EPS I and EPS II production was necessary to enable the high-efficiency removal of cells from culture supernatants and to facilitate the concentration of proteins. Therefore, strains RmAR1157 (Rm2011 *expA1exoY*) and SmLM8311 (Rm2011 *expA3exoYmucR*) were used to determine if ExpE1 is secreted. Detection of ExpE1 in the supernatant of *S. meliloti* strains was additionally complicated by proteolysis. Several bands migrating at sizes lower than 30 kDa reacted with the anti-ExpE1_{C-term} antibody (data not shown). This effect was enormously reduced by adding a protease-inhibitor cocktail to the culture supernatant immediately after removal of the cells.

RmAR1157 grown under phosphate-limiting conditions was assayed for the presence of ExpE1 in cells and supernatant using the anti-ExpE1_{C-term} antibody. The result indicated that ExpE1 is present both in the cell extract (Fig. 3a, lane 1) and the supernatant (Fig. 3b, lane 1). This implies that ExpE1 is secreted into the growth medium.

Some proteins like endoglycanases and cellulases from *Rhizobium leguminosarum* and *Azorhizobium caulinodans* secreted by type I secretion systems were found to be cell-surface associated (Geelen *et al.*, 1995; Mateos *et al.*, 1992). Therefore, we investigated whether ExpE1 can also become attached to the outer membrane of the cell. Preparation of outer-membrane proteins from RmAR9007 TD101 (Rm2011 *exoYmucR*) was carried out according to the protocol described by Poxton *et al.* (1985). Using the anti-ExpE1_{C-term} antibody ExpE1 was not detected in the outer-membrane fraction, but in the cytoplasmic fraction (data not shown).

Since ExpE1 is predicted to be secreted via a signal

peptide-independent pathway, it was tested whether this protein is secreted via a transport system involving ExpD1 and ExpD2 that display homologies to components of type I protein-secretion complexes (Becker *et al.*, 1997). The double mutants SmLM9815 (Rm2011 *ΔexpD1exoY*) and SmLM9831 (Rm2011 *ΔexpD2exoY*) grown under phosphate-limiting conditions were tested for the presence of ExpE1 in cells and supernatants. Immunodetection showed the absence of ExpE1 in the growth medium (Fig. 3b, lanes 2 and 3) of both mutants, indicating that ExpE1 is secreted by a transporter system involving ExpD1 and ExpD2. The protein was found in the cell extract of SmLM9831 (Rm2011 *ΔexpD2exoY*) (Fig. 3a, lane 3), but curiously only a very weak signal at the detection limit was observed in the cell extract of SmLM9815 (Rm2011 *ΔexpD1exoY*) (Fig. 3a, lane 2). The β -galactosidase activity of a translational *expE1-lacZ* fusion expressed in the wild-type background was comparable to the values obtained in the *expD1* or *expD2* mutant. This implies that the deletions in *expD1* and *expD2* did not influence the transcription or translation of *expE1*. ExpE1 was not detected in the cells or the supernatant of SmLM9835 (Rm2011 *ΔexpE1-exoY*) (Fig. 3a and 3b, lanes 4).

To restore the ability of SmLM9835 to express ExpE1, pLM91-1 and pLM93-2 carrying the *expE1* gene under the control of its own promoter were transferred to SmLM9835. Strain SmLM9901 contained the replicating plasmid pLM91-1 whereas strain SmLM9903 carried plasmid pLM93-2 integrated into the genome. The ExpE1 protein was detected in cell extracts and culture supernatants of both strains (Fig. 3a, b, lanes 5 and 6). The introduction of the multicopy plasmid pLM91-1 in SmLM9815 (SmLM9906) resulted in the detection of ExpE1 in the cell extract, but not in the supernatant (Fig. 3a, b, lanes 7). Since a very small amount of ExpE1 protein was detected in the cell fraction of SmLM9815, accumulation of ExpE1 in this mutant might only occur if it is overexpressed. Secretion of ExpE1 was also assayed in the deletion mutants carrying additionally an *exoY* mutation and a *mucR* mutation that stimulated the expression of the *exp* genes. The results of ExpE1 detection in these triple mutants were consistent with those described above, with the exception of strain SmLM9828 (Rm2011 *ΔexpD1exoYmucR*), in which ExpE1 was detected in the cells (data not shown).

ExpD1, ExpD2 and ExpE1 are required for the synthesis or secretion of EPS II

To study the involvement of the secreted protein ExpE1 and its transport system in EPS II secretion, *S. meliloti* strains were used that carried mutations in *exoY* and *mucR* and were grown in low-phosphate medium. Under these conditions, *exp* genes are maximally expressed (Rüberg *et al.*, 1999). Using gel-filtration chromatography, HMM and LMM fractions present in the culture supernatants of strains carrying deletions in *expE1*, *expD1* and *expD2* were separated. No HMM fraction was detected in the culture supernatants of

Table 2. Analysis of culture supernatants by gel-filtration chromatography and sugar content

Standard deviations were equal to or less than 10% and 4% for the determination of the total amount of extracellular carbohydrates in glucose equivalents and the ratio of glucose to galactose, respectively. ND, Not determined.

Strain	Glucose equivalents (mg l ⁻¹)	HMM fraction		LMM fraction	
		Present?	Glc/Gal (%)	Present?	Glc/Gal (%)
RnAR9007 TD101	397	+	51/49	+	ND
SmLM9828	48	—	ND	+	100/0
SmLM9832	534	—	ND	+	100/0
SmLM9836	44	—	ND	+	100/0
SmLM9904	312	+	51/49	+	ND
SmLM9905	320	+	52/48	+	ND
SmLM9907	435	+	53/47	+	ND
SmLM9908	442	+	54/46	+	ND
SmLM8311	17	—	ND	+	100/0
SmLM8311/SmLM9836*	31	—	ND	+	100/0

* Co-cultivation experiment. Values given refer to both strains together.

expE1, *expD1* and *expD2* deletion mutants indicating the absence of extracellular HMM-EPS II (Table 2).

To exclude the possibility of a polar effect of the *expE1* deletion on the transcription of the *expE* genes located downstream of *expE1* (Becker *et al.*, 1997), a promoterless *lacZ*–Gm interposon (Becker *et al.*, 1995) was inserted into the *expE7* gene of mutant SmLM9909 (Rm2011 $\Delta expE1mucR$). The β -galactosidase activities displayed by the resulting strain and the control strain carrying the *expE7*–*lacZ* transcriptional fusion and a mutation in *mucR* were comparable. Since promoter-mapping experiments indicated that *expE7* is exclusively transcribed from a promoter upstream of *expE1* (Becker *et al.*, 1997), this result implies that the *expE1* deletion had no influence on the transcription of the *expE* operon in the *mucR* mutant background. To confirm that the absence of HMW EPS II in SmLM9836 (Rm2011 $\Delta expE1exoYmucR$) was due to the deletion of the *expE1* gene and not caused by a second-site mutation, a complementation strategy was used in which pLM93-2 containing *expE1* was integrated into the genome of SmLM9836 (Rm2011 $\Delta expE1exoYmucR$) by a single homologous recombination event. Depending on whether the recombination occurred upstream or downstream of the *expE1* deletion, transcription of the whole *expE* operon was driven by the *expE1* promoter located in front of the *expE1* deletion (SmLM9904) or in front of the *expE1* wild-type gene (SmLM9905), respectively. Strains with both possible genomic structures regained the ability to produce HMW EPS II, providing additional evidence that the *expE1* deletion had no influence on the expression of the *expE* operon. Using *lacZ* transcriptional fusions no vector promoter that might have contributed to the transcription of the *expE* operon in the merodiploid strains was identified. Complemen-

tation of the *expD1* or *expD2* deletion mutants by integration of pLM811-1 carrying the *expD1* and *expD2* wild-type genes into the genome of SmLM9828 (Rm2011 $\Delta expD1exoYmucR$) or SmLM9832 (Rm2011 $\Delta expD2exoYmucR$) also restored the ability of *S. meliloti* to produce EPS II (Table 2).

The total sugar content of the EPS fractions was determined (Table 2). A high sugar content in the culture supernatant correlated with the detection of a HMW fraction with the exception of strain SmLM9832. In the culture supernatant of this strain a LMW fraction was exclusively detected. In this LMW fraction glucose but no galactose was detected, implying that strain SmLM9832 secreted no LMW EPS II and might have secreted a high amount of cyclic glucans. In the culture supernatants of strains SmLM9828 and SmLM9836 a LMW fraction containing a low amount of total sugars was exclusively detected (Table 2). To confirm that the HMW fraction produced by the strains secreting ExpE1 contained EPS II, the glucose and galactose content was quantified. The HMW fractions from RmAR9007 TD101, SmLM9836::pLM93-2, SmLM9828::pLM811-1 and SmLM9832::pLM811-1 contained glucose and galactose in the ratio of approximately 1:1 (Table 2), indicating that these fractions contained EPS II. Glucose, but no galactose was detected in the LMW fractions of strains SmLM9828 and SmLM9836, indicating the possible presence of cyclic glucans but no LMW EPS II as was already suggested for the LMW fraction of mutant SmLM9832 (Table 2).

Since ExpE1 might function extracellularly in EPS II biosynthesis or secretion, we tested if ExpE1 could be supplied *in trans* to stimulate HMW-EPS II production by mutant SmLM9836. This strain, which is unable to

produce EPS II due to the deletion of *expE1* was co-cultivated with SmLM8311, which secretes ExpE1 but does not produce EPS II due to a mutation in *expA3* encoding a glycosyltransferase. After 10 d growth in low-phosphate medium, no HMW fraction was detected in the culture supernatant (Table 2). This was also confirmed by the low sugar content in the culture supernatant.

DISCUSSION

The *expE1* gene situated in the *exp* gene cluster of *S. meliloti* 2011 encodes the ExpE1 protein that was shown to be secreted and to bind calcium ions. These results are in accordance with similarities of ExpE1 to other secreted proteins like toxins, metalloproteases or NodO (Becker *et al.*, 1997; Delepelaire & Wandersman, 1990; Economou *et al.*, 1990; Mackman *et al.*, 1986). In addition to the Ca^{2+} -binding domains characteristic of these proteins, they contain amphipathic α -helices close to the C terminus that are thought to be involved in secretion (Duong *et al.*, 1992, 1994; Ghigo & Wandersman, 1992).

An amphipathic α -helix close to the C terminus and 15 Ca^{2+} -binding domains were predicted for ExpE1 (Becker *et al.*, 1997). The repeat sequence GGXGXDXUX (where U is any hydrophobic amino acid) was found in most of the members of a family of bacterial proteins that are secreted by type I secretion systems (Binet *et al.*, 1997). The three-dimensional structure of several of these secreted proteins demonstrated that the repeated motif binds Ca^{2+} in a parallel β -roll structure (Baumann *et al.*, 1993; Baumann, 1994). Baumann *et al.* (1993) suggested that the Ca^{2+} -binding regions may have a role in folding of the molecule after transmembrane translocation. In the absence of Ca^{2+} this structure is unstable and could facilitate membrane translocation of the polypeptide in an unfolded form. The presence of Ca^{2+} in the extracellular medium could induce the polypeptide to fold in the right tertiary structure. Another possible role for the calcium domains was proposed for haemolysin A and the adenylate cyclase toxin from *Bordetella pertussis* (Bakás *et al.*, 1998; Rose *et al.*, 1995). In both cases, it was suggested that binding of Ca^{2+} may induce a conformational change that would result in surfacing hydrophobic portions of the protein. The increased hydrophobic surface probably favours cell-membrane binding that appears to be essential for the lytic process.

The M_r estimated by SDS-PAGE for ExpE1 or $(\text{His})_6\text{ExpE1}$ showed an abnormal value. In both cases, this value was 8–10 kDa higher than the M_r predicted by its amino acid sequence. The discrepancy in the predicted and observed M_r in SDS-PAGE was also reported for NodO from *R. leguminosarum*, a protein that shows some sequence similarity with ExpE1 (Economou *et al.*, 1990) and other acidic proteins (Kaufman *et al.*, 1984). Size fractionation of the $(\text{His})_6\text{ExpE1}$ by gel filtration indicated that this protein purifies as a dimer. The same

observation was also made for NodO (Economou *et al.*, 1990).

expD1 and *expD2* were required for the secretion of ExpE1. Since ExpD1 and ExpD2 are homologous to ABC transporters and membrane-fusion proteins, respectively, of type I secretion systems, we propose that ExpE1 is secreted by a type I secretion system involving ExpD1 and ExpD2. In a genetic background allowing strong expression of *expE1*, ExpE1 accumulated in the cell if *expD1* or *expD2* were deleted. Under conditions allowing a medium level expression of *expE1* this was also the case in an *expD2* mutant but not in an *expD1* mutant. Since expression of an *expE1-lacZ* translational fusion was not affected in the *expD1* deletion mutant, the low amount of ExpE1 detected might have resulted from degradation. Hence, the absence of ExpD1 may destabilize or its presence may stabilize ExpE1 in the cell. It is possible that ExpE1 was also degraded in an *expD1* mutant when it was overexpressed, but due to the high amount of ExpE1 produced, this degradation might not have occurred at a relevant level.

The *expE1*, *expD1* and *expD2* genes were shown to be required for the synthesis or secretion of EPS II. Since secretion of ExpE1 required *expD1* and *expD2*, ExpD1 and ExpD2 may be indirectly required for EPS II production. A direct involvement of ExpD1 and ExpD2 in EPS II production independent of their function in secretion of ExpE1 might be the secretion of EPS II or its precursors. An involvement of type I secretion systems in the secretion of polysaccharides was described for *S. meliloti* and *Agrobacterium tumefaciens* cyclic (1,2)- β -glucans and *E. coli* K1 capsular polysaccharide (Bliss & Silver, 1996; Breedveld & Miller, 1994). The NdvA and ChvA proteins from *S. meliloti* and *A. tumefaciens* share homologies with bacterial ABC transporter proteins, and mutants in these genes did not secrete cyclic (1,2)- β -glucans to the periplasmic compartment and to the extracellular medium. In *E. coli* K1, sialic acid is synthesized and polymerized in the cytoplasm, and linking of the synthetic and transport pathways is possibly performed by the proteins KpsC and KpsS that may 'escort' the polySia chains to the transport machinery. This machinery is composed of a type I secretion system comprising KpsM, KpsT and KpsE, that together with the periplasmic protein KpsD and an unknown outer-membrane protein, are responsible for the translocation of the capsular polysaccharide across the inner and outer membranes. Since ExpD1 and ExpD2 resemble components of type I protein-secretion systems and we have demonstrated that these two proteins are required for secretion of ExpE1, it appears rather unlikely that the ExpD1/ExpD2 type I transporter system is additionally involved in the secretion of a polysaccharide or polysaccharide precursors.

Supplying ExpE1 *in trans* in a co-cultivation experiment did not restore the ability of a *S. meliloti expE1* mutant to produce extracellular EPS II. The ExpE1 protein might not be stable enough in the culture medium to correct the deficiency of an *expE1* mutant in EPS II

synthesis or to fulfil its function ExpE1 has to be synthesized and secreted by the cell that synthesizes EPS II. ExpE1 is similar to NodO from *R. leguminosarum* which was found to insert into liposomes and when added to planar lipid bilayers, formed cation-selective channels that allowed the movement of monovalent cations across the membrane (Sutton *et al.*, 1994). Sutton *et al.* (1994) suggested that the role of NodO in nodulation might be the formation of pores in the plant plasma membrane. ExpE1 and NodO mainly consist of the nonapeptide repeats and the C-terminal 85 aa of ExpE1 display no similarities to NodO. Therefore, functions of these two proteins cannot necessarily be suggested based on these sequence similarities.

Since ExpE1 was not detected in the outer-membrane fraction, it seems to be rather unlikely that ExpE1 forms pores in this membrane that are involved in the secretion of EPS II. On the other hand, for technical reasons ExpE1 was recovered from cells and supernatants of *S. meliloti* strains that were unable to produce EPS I and EPS II. Therefore, we cannot exclude the possibility that the localization of ExpE1 in the wild-type depends on the presence of EPS I or EPS II. Weisgerber & Troy (1990) reported that immunoprecipitated polySia obtained from labelled *E. coli* K1 membranes was found to be associated with a protein of 20 kDa which has an unknown function. Hence, the hypothesis that ExpE1 mediates the secretion of EPS II by binding to the nascent EPS II chain might be conceivable. Although it was shown that ExpE1 is required for EPS II synthesis or secretion, the role of this protein and its transport system in EPS II production remains unknown, and further studies will be required to understand their function.

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