An *exoB* mutant of *Rhizobium* sp. is effective in indeterminate nodules of *Hedysarum coronarium*

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A *Rhizobium* sp. (*Hedysarum coronarium*) calcofluor dark (Cal⁻) mutant, named Cal10, was obtained following Tn5mob-insertion mutagenesis. It is affected in the synthesis of exopolysaccharide and presents an altered lipopolysaccharide that is not recognized by a polyclonal antibody against the lipopolysaccharide of the parental strain. The residual exopolysaccharide obtained from the mutant lacks galactose and the high-molecular-mass acidic fraction. This mutant was complemented by plasmid pD56 that restores the production of exopolysaccharide, the alteration of lipopolysaccharide and the Cal⁺ phenotype. The data presented indicate that the gene in which the mutant is defective is homologous to the *exoB* gene of *Rhizobium meliloti* and fails to synthesize UDP-glucose 4'-epimerase. The Cal10 mutant was Fix⁺ on *H. coronarium* (sulla) although it develops an indeterminate type of nodule, indicating that exopolysaccharide is not essential for a successful nodulation in this symbiotic association.

Keywords: *Rhizobium* sp., nodulation, symbiosis, exopolysaccharide, *exoB*

INTRODUCTION

Rhizobium strains produce exopolysaccharide (EPS). Several authors have demonstrated that EPS production is necessary for successful nodulation on legumes that develop an indeterminate type of nodule (nodules with a meristematic zone) such as alfalfa, peas or clovers (Borthakur *et al.*, 1986; Chakravorty *et al.*, 1982; Leigh *et al.*, 1985; Canter Cremers *et al.*, 1990). However, EPS does not seem to be essential for nodulation on legumes with determinate nodules without a meristematic zone such as beans or soybeans (Diebold & Noel, 1989; Ko & Gayda, 1990). Hotter & Scott (1991) demonstrated that *R. loti* EPS mutants induce Fix⁺ nodules on the determinate nodulating host *Lotus pedunculatus* and Fix⁻ on the indeterminate nodulating host *Leucaena leucocephala*.

Lipopolysaccharide (LPS) also seems to be implicated in symbiosis. LPS is necessary for infection thread development in *R. leguminosarum* biovar *phaseoli* (Noel *et al.*, 1986; Cava *et al.*, 1989). A non-nodulating mutant or a mutant that nodulates very slowly has been described in *Bradyrhizobium japonicum* strains with altered LPS (Puvanesarajah *et al.*, 1987; Stacey *et al.*, 1982). However, Clover

et al. (1989) have described several LPS mutants of *R. meliloti* that are not defective in symbiosis.

The *exoB* mutants of *R. meliloti* and *R. leguminosarum* biovar *viciae* are pleiotropic. They are defective in the synthesis of both EPS and LPS and induce empty ineffective nodules (Leigh *et al.*, 1985; Leigh & Lee, 1988) or only some root hair deformation and rare abortive infection threads (Canter Cremers *et al.*, 1990). However, several *exoB* mutants of *Bradyrhizobium japonicum* are altered in their EPS but not LPS and form effective nodules on soybeans (Parniske *et al.*, 1993) where a determinate type of nodule develops.

The *exoB* gene encodes a UDP-glucose 4'-epimerase in *R. leguminosarum*, *R. meliloti* and *B. japonicum* (Canter Cremers *et al.*, 1990; Buendía-Clavería *et al.*, 1991; Parniske *et al.*, 1993).

In this work we describe an *exoB* mutant of *Rhizobium* sp. that induces effective nodules on its host plant *Hedysarum coronarium*, indicating that EPS is not essential in all symbiotic interactions in indeterminate nodule-type legumes.

Abbreviation: EPS, exopolysaccharide.

Table 1. Bacterial strains and plasmids

Strain and plasmid	Characteristics*	Reference
Strains		
<i>E. coli</i> S17-1	Tp ^r , Sm ^r (pSUP5011).	Simon (1984)
<i>Rhizobium</i> sp. (<i>H. coronarium</i>)		
IS123	Wild type.	Espuny <i>et al.</i> (1987)
RB16	Spontaneous Sm ^r derivative of IS123.	Espuny <i>et al.</i> (1987)
RJ84	RB16 harbouring the pSym plasmid tagged with Tn5 <i>mob</i> .	Ollero <i>et al.</i> (1989)
RB19	Spontaneous Rif ^r derivative of IS123.	Ollero <i>et al.</i> (1989)
Cal10	Tn5 <i>mob</i> induced calcofluor dark mutant of RB19. Km ^r , Rif ^r .	This study
Plasmids		
pSUP5011	Tn5 <i>mob</i> Ap ^r , Cm ^r .	Simon (1984)
pRK2013	Helper. Km ^r .	Ditta <i>et al.</i> (1980)
pJB3JI	Helper. Tc ^r , Ap ^r .	Brewin <i>et al.</i> (1980)
pD56	pLAFR1 cosmid from a <i>R. meliloti</i> gene library complementing mutants <i>exoB,Z,Q,F,Y,X</i>	Long <i>et al.</i> (1988)
pEX312	pLAFR1 cosmid from a <i>R. meliloti</i> gene library complementing mutants <i>exoZ,Q,F,Y,X,H,K,L,A,M,N,P</i>	Long <i>et al.</i> (1988)
pEX154	pLAFR1 cosmid from a <i>R. meliloti</i> gene library complementing mutants <i>exoH,K,L,A,M,N,P</i>	Leigh <i>et al.</i> (1987)

* Resistance to ampicillin (Ap^r), chloramphenicol (Cm^r), kanamycin (Km^r), rifampicin (Rif^r), streptomycin (Sm^r), tetracycline (Tc^r) and trimethoprim (Tp^r).

METHODS

Strains, plasmids, media and bacterial matings. Bacterial strains and plasmids are listed in Table 1. *Escherichia coli* strains were grown on LB medium and *Rhizobium* on TY (Beringer, 1974) or YM (Vincent, 1970) media. Antibiotics were added to media at the following concentrations (µg ml⁻¹): kanamycin (25) and tetracycline (10) for *E. coli*; and kanamycin (60), tetracycline (10), streptomycin (400), rifampicin (50) and nalidixic acid (20) for *Rhizobium*. Conditions for plasmid transfer by conjugation were performed as described by Buchanan-Wollaston *et al.* (1980). In triparental matings the helper plasmids pRK2013 or pJB3JI were used.

Isolation of the Cal10 mutant. The parental strain RB19, which produces a calcofluor-binding EPS, was mutagenized with transposon Tn5*mob* by means of the suicide vector pSUP5011. The resulting bacterial clones were screened by plating on TY medium supplemented with rifampicin, kanamycin and 0.02% (w/v) calcofluor (Fluorescent Brightener 28, Sigma). A calcofluor dark colony was selected for further studies.

DNA isolation and manipulation. Plasmid profiles of rhizobia strains were visualized according to the agarose gel electrophoresis method of Eckhardt described previously (Espuny *et al.*, 1987). Total *Rhizobium* DNA was isolated and purified by the method of Meade *et al.* (1982) and cut with *Eco*RI (Boehringer-Mannheim). A 870 bp *Pst*I fragment of the kanamycin resistance gene of Tn5 was used as a probe for hybridization, with digoxigenin labelling and Lumi-Phos detection (Boehringer-Mannheim).

Isolation and analysis of EPS. Bacteria were grown in YM broth for 7 d at 28 °C in a rotary shaker. The EPS was isolated by precipitation from the culture supernatant with 2 vols acetone or 4 vols cold ethanol, or by the method of Diebold & Noel (1989). The EPS was analysed by gel filtration chromatography, using a Sepharose 4B column. The hexose content was measured by the anthrone method (Spiro, 1962). Uronic acid was assayed by reaction with *m*-hydroxydiphenyl (Blumenkrantz & Asboe-Hanson, 1973). To determine the EPS composition, it was first hydrolysed in 1 M HCl acid for 5 h at 100 °C and then analysed by thin layer chromatography (TLC) using cellulose plates F-254 (Merck). The solvent used was butanol/pyridine/water (6:4:3, by vol.). The plates were developed with 5% triphenyltetrazolium chloride in methanol and 0.25 M NaOH.

Production of anti-LPS polyclonal antibodies. A rabbit was inoculated intramuscularly with 1.5 mg LPS isolated according to Bellogín *et al.* (1984) and emulsified in 0.5 ml Freund's complete adjuvant. Furthermore, subcutaneous inoculations with 1 mg LPS emulsified in 0.5 ml Freund's incomplete adjuvant were carried out according to the immunization scheme described in detail elsewhere (Sánchez-Palazón & Rodríguez-Burgos, 1993). The antiserum obtained was assayed against LPS by the double diffusion technique.

Acrylamide gel electrophoresis for LPS analysis. Bacterial cells were harvested after growth to stationary phase in TY broth by centrifugation of 1 ml samples. Pelleted cells were resuspended in 300 µl Laemmli solubilization buffer (Laemmli, 1970), treated with proteinase K (50 µg ml⁻¹) according to Sindhu *et al.* (1990)

and subsequently separated using SDS-PAGE (15% acrylamide). The LPS profile was visualized by a silver staining procedure described by Cava *et al.* (1989).

Immunoblotting. The gels were electroblotted for 1 h at 125 mA to nitrocellulose sheets in 48 mM Tris, 39 mM glycine, 20% (v/v) methanol and 0.0375% (w/v) SDS using a semi-dry LKB Multiphor II apparatus. Then the sheets were immunostained with the polyclonal antibody (dilution 1:25) using a peroxidase-conjugated IgG as the secondary antibody and developed using the Amersham ECL system, according to the manufacturer's instructions.

Enzyme assay for UDP-glucose 4'-epimerase. Bacteria were grown in YM medium, collected by centrifugation, and disrupted by sonication. Cell-free extracts were obtained by centrifugation at 26000 *g* for 1 h at 0 °C. UDP-glucose 4'-epimerase was determined by the one-step method as described by Fukasawa *et al.* (1980). Protein concentration of crude extracts was determined by the Lowry method.

Plant tests. Seeds of *Hedysarum coronarium* ecotype Menorca, were sterilized and germinated, and plants were grown and inoculated as described previously (Espuny *et al.*, 1987).

Light microscopy. Nodules 4 weeks old were removed, treated by the method of Hotter & Scott (1991) and embedded in a Spurr's resin. For microscopy observations, 0.5 µm sections were stained with toluidine blue (Hotter & Scott, 1991).

RESULTS

Isolation of calcofluor dark strains

A calcofluor dark colony (named Cal10) was obtained after *Tn5mob* mutagenesis. Wild-type *Rhizobium* sp. RB19 colonies on YM medium were highly mucoid in appearance but the *exo* mutant Cal10 produced colonies that were non-mucoid on YM, and did not produce acetone-precipitable EPS.

Rhizobium sp. RB19 harbours three plasmids with molecular masses of about 160, 230 and > 600 MDa (Ollero *et al.*, 1989). To see if the transposon *Tn5mob* was inserted within these plasmids, a blotted Eckhardt gel was hybridized using a *Tn5* probe. A positive hybridization signal was detected from RJ84, which harbours *Tn5mob* in the symbiotic plasmid, but was not detected from plasmid DNAs from the Cal10 mutant. Positive signals were detected around the agarose gel wells where large chromosomal DNA fragments were retained (data not shown).

Southern hybridization of total DNA digested by *Eco*RI revealed that the Cal10 mutant harbours one copy of the transposon located in an *Eco*RI fragment of 13.4 kb (data not shown). As the transposon *Tn5mob* is about 7.6 kb in size and *Eco*RI does not cut the transposon, the insertion is located in a Cal10 chromosomal DNA fragment of about 5.8 kb.

Analysis of EPS

The Cal10 mutant did not produce acetone-precipitable EPS; however, it was possible to isolate some EPS using the Diebold & Noel (1989) extraction method. It was estimated that parental strain RB19 produces about 0.0013 pg per viable cell and the Cal10 mutant about 0.00015 pg per viable cell.

EPS from the parental strain was separated into two peaks by Sepharose 4B gel filtration, a high-molecular-mass acidic EPS fraction and a low-molecular-mass neutral EPS fraction (Fig. 1a). In contrast, the EPS of the Cal10 mutant resulted in one peak of low-molecular-mass neutral EPS (Fig. 1b).

In the hydrolysed EPS of parental strain RB19, spots indicating the presence of glucose and galactose were detected on TLC plates. The EPS of the Cal10 mutant lacks the galactose.

Complementation of the Cal10 mutant by *R. meliloti* *exo* genes

Several cosmid clones containing *exo* genes of *R. meliloti* have been described (Leigh *et al.*, 1987; Long *et al.*, 1988). In this work cosmids pD56, pEX154 and pEX312, which together harbour all described *exo* genes of *R. meliloti* were used for complementation studies of the Cal10 mutant.

Only plasmid pD56, which harbours *exoX*, *exoY*, *exoF*, *exoQ*, *exoZ* and *exoB* was able to complement the Cal10 mutant. The complemented mutant was Cal⁺, mucoid in YM medium and produced high-molecular-mass acidic

Fig. 1. Elution profile of a Sepharose 4B gel filtration column of EPS from parental strain RB19 (a) and the Cal10 mutant (b). Hexose (□) was measured by the anthrone method (A_{620}), and uronic acid (△) was assayed by reaction with *m*-hydroxydiphenyl (A_{520}). In (a) the uronic acid peak is coincident with the first hexose peak. In (b) uronic acid was not detected.

Fig. 2. (a) LPS profiles of *Rhizobium* sp. (*H. coronarium*) strains visualized by periodate oxidation and silver staining after electrophoresis on 15% acrylamide (w/v) gels containing SDS. Lanes: 1, RB19; 2, Cal10; 3, Cal10(pD56). (b) Immunoblot, incubated with antiserum against RB19 LPS, of a duplicate gel corresponding to the one shown in (a).

Fig. 3. UDP-glucose 4'-epimerase activity of *Rhizobium* sp. (*H. coronarium*) strains RB19, Cal10 and Cal10(pD56).

EPS. Plasmid pEX312 harbours all these *exo* genes except *exoB*, but does not complement Cal10, indicating that Cal10 is mutated in the *exoB* gene.

Characterization of the Cal10 mutant

The *exoB* mutants have been described as pleiotropic and are affected in synthesis of both EPS and LPS (Canter Cremers *et al.*, 1990). The LPS pattern of parental *Rhizobium* sp. strain RB19 consists of two parts, LPSI and LPSII (Fig. 2a). According to other authors (Sindhu *et al.*, 1990), LPSI is presumably the entire LPS (lipid A, core and O-antigen) and LPSII, that migrated just behind the solvent front, consists of only lipid A and core. Mutant Cal10 produced a LPS, which also consists of two parts, but both migrated faster than LPSI and LPSII of

Fig. 4. Light microscopy photograph of a longitudinal section of a nitrogen-fixing nodule induced by the parental strain RB19. Nodule meristem (NM), nodule cortex (NC), invasion zone (IZ), nodular vascular bundles (arrows). Bar, 100 µm. Nodules induced by the Cal10 mutant exhibited the same structure.

the wild-type strain (Fig. 2a). The complemented Cal10(pD56) strain had the same LPS profile as the parental RB19 strain (Fig. 2a).

SDS-PAGE blots of LPS were incubated with antiserum against the parental strain LPS. The Cal10 mutant LPS was not recognized by this antiserum, while the LPS of the complemented Cal10(pD56) strain, like the parental strain LPS, was recognized (Fig. 2b).

The Cal10 *exo::Tn5mob* mutation was transferred by conjugation to strain RB16 (Sm^r derivative of wild-type *Rhizobium* sp. IS123). All Km^r transconjugants were non-mucoid, Cal⁻, and had the same LPS profile as Cal10. Therefore, the *exo* mutation was at least closely linked to the Tn5mob insertion.

To confirm that the Cal10 mutant fails to synthesize active UDP-glucose 4'-epimerase, an enzymic assay for this enzyme was carried out using cell-free extracts prepared from *Rhizobium* sp. strains RB19, Cal10 and Cal10(pD56). The results showed that the Cal10 mutant lacks this enzymic activity (Fig. 3), while pD56 restores activity at an enhanced level that possibly reflects a higher copy number of the *exoB* gene.

Cal10, like the parental strain, grows on galactose as the sole carbon source.

Symbiotic properties of the *exoB* mutant

Rhizobium sp. strain IS123 induces cylindrical or branched nodules on *H. coronarium* (sulla). Light microscopic examination of the nodules showed a clearly visible meristematic zone (Fig. 4), typical of an indeterminate type of nodule.

The *exoB* mutant induced, on sulla, nodules that were comparable in number and morphology to those induced by wild-type strain RB19. Furthermore, mutants had the same nodulation kinetic and fixed nitrogen at the same

rate as the wild-type strain. Bacteria recovered from nodules induced by mutants were phenotypically the same with regard to EPS production, LPS profile and antibiotic resistance as the original mutant Cal10.

DISCUSSION

In this paper we describe an *exoB* mutant of *Rhizobium* sp. (*H. coronarium*) which has been genetically and enzymically characterized. Like other *exoB* *Rhizobium* mutants, Cal10 is deficient in the synthesis of UDP-glucose 4'-epimerase.

This mutant is pleiotropic and both fails to synthesize EPS and presents an altered LPS, as do the *exoB* mutants described in *R. meliloti* and *R. leguminosarum* biovar *viciae* (Leigh & Lee, 1988; Canter Cremers *et al.*, 1990). The mutant is not mucoid and the residual EPS, isolated using the method described by Dielbold & Noel (1989), lacks galactose residues and the acidic component (Fig. 1). These results suggest that the residual EPS isolated from the Cal10 mutant is a neutral β -glucan and the galactose must be a component of the acidic EPS fraction.

As can be seen in Fig. 2, the Cal10 mutant presents a LPS profile similar to the wild type but both LPSI and LPSII migrate faster than those corresponding to the parental strain. The Cal10 LPS is clearly an altered form of the wild-type LPS because it is not recognized by the anti-wild-type LPS antibody.

In this paper we show that Cal10 lacks UDP-glucose 4'-epimerase, the enzyme that interconverts UDP-glucose and UDP-galactose. This explains the lack of galactose residues in the Cal10 EPS. Probably, the alteration in the Cal10 LPS could be a consequence of the lack of galactose residues. This would affect the polymerization degree of the LPS and for this reason the bands corresponding to LPSI and LPSII migrate faster than those corresponding to the wild-type strain. The absence of galactose residues in Cal10 LPS could also explain the incapacity of the antibody to recognize this LPS, indicating that galactose could be part of the epitope.

Like other *Rhizobium* *exoB* mutants, Cal10 is able to grow on galactose as sole carbon source, in contrast to *E. coli* *galE* mutants, where galactose as the unique carbon source is lethal (Adhya, 1987). Thus, *Rhizobium* sp. (*H. coronarium*) also makes use of the De Ley-Doudoroff pathway for the conversion of galactose as do *R. leguminosarum* biovar *trifolii* (Ronson & Primrose, 1979) and *R. meliloti* (Arias & Cervenansky, 1986).

It has been reported that the *exoB* gene is necessary for successful nodulation on legumes that develop an indeterminate type of nodule (Leigh *et al.*, 1985; Leigh & Lee, 1988; Canter Cremers *et al.*, 1990), but does not seem to be essential for nodulation on legumes with a determinate type of nodule (Parniske *et al.*, 1993). However, we report in this paper an *exoB* mutant of *Rhizobium* sp. (*H. coronarium*) that induces effective nodules on sulla, a legume that develops an indeterminate type of nodule (Fig. 4).

Williams *et al.* (1990) reported that the symbiotic defect

(Fix⁻) of *R. meliloti* *exo* mutants can be suppressed by the *lpsZ*⁺ gene, which is present in Rm41 but not in SU47. Therefore the *exoB* mutants of Rm41 are Fix⁺. It is possible to suppose that the *Rhizobium* sp. strain RB19 harbours a *lpsZ*⁺-like gene, and this could explain the Fix⁺ phenotype of our *exoB* mutant. Further work will be carried out to explore this possibility.

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