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Alteration of O-specific polysaccharide structure of symbiotically defective *Mesorhizobium loti* mutant 2213.1 derived from strain NZP2213

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***Mesorhizobium loti* mutant 2213.1 derived from the wild-type strain NZP2213 by Tn*5* mutagenesis showed impaired effectiveness of symbiosis with the host plant *Lotus corniculatus* (Turska-Szewczuk *et al*., 2007 *Microbiol Res*, in press). The inability of lipopolysaccharide (LPS) isolated from the mutant 2213.1 strain or de-O-acetylated LPS of the parental cells to inactivate phage A1 particles implicated alterations in the LPS structure. The O-specific polysaccharide of the mutant was studied by chemical analyses along with ^{1}H and ^{13}C NMR spectroscopy, which clearly confirmed alterations in the O-chain structure. 2D NMR data showed that the mutant O-polysaccharide consists of a tetrasaccharide repeating unit containing non-substituted as well as O-acetylated or O-methylated 6-deoxytalopyranose residues. Additionally, an immunogold assay revealed a reduced number of gold particles on the mutant bacteroid cell surface, which could result from both a diminished amount of an O-antigenic determinant in mutant LPS and modifications of structural epitopes caused by alterations in O-acetylation or O-methylation of sugar residues. Western immunoblot assay of alkaline de-O-acetylated lipophilic *M. loti* NZP2213 LPS showed no reactivity with homologous serum indicating a role of O-acetyl groups in its O-specificity.**

Keywords: O-specific polysaccharide, bacterial polysaccharide structure, *Mesorhizobium loti*, symbiosis, 6-deoxytalose

INTRODUCTION

The symbiotic interaction between rhizobial bacteria and leguminous plants is in general a species-specific process that requires a continuous signal exchange resulting in the development of root nodules where the bacteria differentiated into bacteroids are able to fix atmospheric nitrogen. Nod factors together with additional microbial signals such as exopolysaccharides and secreted proteins allow bacteria attached to root hairs to enter the host cells *via* an endocytosis-like process and then establish a chronic intracellular infection within the plant cells (Campbell *et al*., 2002). The success of colonization and survival within the host also requires that rhizobia evade plant defence responses, a process in which among other factors bacterial surface polysaccharides are involved (Soto *et al*., 2006). The significance of the O-antigenic portion of lipopolysaccharide to the establishment of a compatible interaction between the bacterial and host cells during the formation of the infection thread and the release of the microsymbionts into the plant cytoplasm has been documented for several *Rhizobium* species (Noel *et al*., 2000; Kannenberg & Carlson, 2001; Janczarek *et al*., 2001). Although little is known of the biological role of specific structural features of the rhizobial LPS, the importance of S-type LPS containing a nearly normal amount of O-antigenic polysac-

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Abbreviations: 6dTal, 6-deoxy-L-talose; Kdo, 3-deoxy-2-octulosonic acid; LPS, lipopolysaccharide; OPS, O-specific polysaccharide; S-RPS, s-form LPS.

charide was precisely documented for *Rhizobium etli — Phaseolus vulgaris* symbiosis during the development of determinate type nodules (Noel *et al*., 2000; 2004). S-form LPS on the bacterial cell surface is crucial to symbiosis but also non-carbohydrate substituents of LPS such as O-acetylation or O-methylation may have an effect on the symbiotic interactions (Lam *et al*., 1992; Noel *et al*., 2004). Therefore, recognition of the structure of O-specific polysaccharides of *Mesorhizobium loti* species seems indispensable for correlating the biological role with specific structural features of the molecule (D'Antuono *et al*., 2005).

Mesorhizobium loti has been described as a microsymbiont of plants classified in the genus *Lotus* (Jarvis *et al*., 1982; 1997). Strain NZP2213 of *M. loti* is a representative of a group of species that form nitrogen-fixing, effective nodules on a limited range of legume hosts, i.e., on *L. corniculatus,* and ineffective nodules on *L. pedunculatus* and *Leucaena leucocephala* (Scott *et al*., 1996). The highly hydrophobic O-chain isolated from phenol-soluble LPS of *M. loti* NZP2213 has been described as a homopolymer composed of α-(1→3)-linked 2-*O*-acetyl-6-deoxy-L-talose residues (Russa *et al*., 1995a; 1995b). The O-specific polysaccharide of *M. huakuii* strain IFO15243T is also built of 6-deoxysugars; however, this O-chain containing 6-deoxytalose and rhamnose has a more hydrophilic character (Choma *et al*., 2000; Choma, 2002).

In this paper, we report the structure of the O-specific polysaccharide of the Tn*5* mutant 2213.1 (Turska-Szewczuk *et al*., 2007) which differs from *M. loti* NZP2213, the parental strain, in its distorted symbiotic behaviour as well as an inability of its LPS to inactivate phage A1 particles.

EXPERIMENTAL PROCEDURES

Bacterial strain, growth, and isolation of lipopolysaccharide and O-polysaccharide. *Mesorhizobium loti* 2213.1 bacteria were cultivated at 28°C in liquid mannitol/yeast extract medium supplemented with kanamycin (30 μg/ml) aerated by vigorous shaking. Cells were pelleted at 10 000 × *g*, washed twice with 0.5 M saline and once with distilled water. The bacterial mass was extracted three times by the hot phenol/water method (Westphal & Jann, 1965). Lipophilic S-type lipopolysaccharide was recovered from the phenol phase and purified by repeated ultracentrifugation at 105 000 × *g* for 4 h as described by Russa *et al*. (1995a). The LPS was then degraded by mild acid hydrolysis with 1.5% acetic acid at 100°C for 2 h. The supernatant containing O-specific PS (OPS), after removal of lipid A by centrifugation, was concentrated and fractionated on a Sephadex G-50 fine column using 1.5% acetic acid as an eluent.

Test for LPS as a phage A1 receptor. The S-type LPSs isolated from *M. loti* NZP2213 wild-type strain (Russa *et al*., 1995a), and from mutant 2213.1 cells as well as chemically modified S-LPS of the parental strain were tested by the phage A1 inactivation test (Turska-Szewczuk & Russa, 2000). The appropriate LPS at a concentration of 1 and 10 μg/ml in SM buffer (Maniatis *et al*., 1982) was mixed with an equal volume of phage A1 containing 3×10^3 pfu/ml. The mixture was incubated at 30°C without shaking for 60 min and tested for virulent phage particles with *M. loti* NZP2213 as an indicator culture as described earlier (Turska-Szewczuk & Russa, 2000).

De-O-acetylation of LPS and heat-killed parental bacteria. Mild alkaline methanolysis of the LPS molecule (Rietschel *et al*., 1972) or of heat-killed bacterial cells was used to examine the effect of de-O-acetylation of O-polysaccharide both in the S-form LPS molecule and in bacterial cells, on adsorption of phage A1 particles as well as on antibody reactivity. LPS (10 mg) was incubated with 0.5 M of sodium methoxide in methanol for 16 h at 20°C (for partial de-O-acetylation for 4 h at 20°C) with stirring. Sedimenting LPS after neutralization with 1 M HCl was dissolved in an appropriate volume of SM buffer or water to obtain a desirable concentration of LPS for the phage inactivation test or an SDS/PAGE analysis, respectively.

Culture of *M. loti* NZP2213 bacteria was washed twice with PBS buffer (pH 7.2) supplemented with 5 mM $MgCl_2$ and the bacteria were killed by boiling for 10 min and freeze-dried. For de-O-acetylation, 5 mg dry mass of bacteria was incubated with 0.5 M sodium methoxide in methanol for 16 h at 20°C. Bacterial pellet was neutralized with 1 M HCl, washed twice in buffer and resuspended in a fresh portion to an appropriate cell density.

Sugar analysis. The polysaccharide was hydrolysed with 2 M trifluoroacetic acid (TFA) for 2 h at 120°C. Liberated monosaccharides were converted into alditol acetates (Russa *et al*., 1995a). The absolute configuration of the sugars was determined by GC analysis of acetylated (–)-2-butyl glycoside derivatives using authentic sugars as standards according to a published method (Gerwig *et al*., 1978). Methylation with trideuteriomethyl iodide was performed according to the Hakomori (1964) method and the products were purified on a Sep-Pak C_{18} cartridge (York *et al*., 1986). The resulting material was subjected to solvolysis in 90% formic acid (80°C, 1 h) (McNeil *et al*., 1982), hydrolysis in 2 M TFA (120°C, 2 h), and then reduction with $NaBD_4$. Partially methylated alditols were converted into acetate derivatives and analysed by GC-MS.

General methods. GC-MS analyses of sugar derivatives were carried out on a Hewlett-Packard gas chromatograph (model HP5890A) equipped with a capillary column (HP-5MS, 30 m × 0.25 mm) and connected to a mass selective detector (MSD model HP 5971). Helium was the carrier gas (0.7 ml/min) and the temperature program was initially 150°C for 5 min, then raised to 310°C at a rate of 5°C/min, final time 20 min.

NMR spectroscopy. ^{1}H and ^{13}C NMR experiments were performed in D_2O solutions with acetone as an internal standard (δ_H 2.225 ppm, δ_C 31.45 ppm). 2D (DQF COSY, NOE, TOCSY) ^{1}H NMR and ^{1}H, ^{13}C ge-HSQC (gradient enhanced-HSQC), and ge-HMBC experiments were carried out on a Varian Unity plus 500 instrument at 60°C using standard Varian software. 1D ^{13}C NMR was obtained with a Bruker DRX-500 Avance spectrometer in D_2O at 60°C.

Immunogold localization of LPS in bacteroids. Twenty-eight-day-old nodules isolated from *Lotus corniculatus* roots inoculated with the mutant 2213.1 and wild-type strains were dehydrated, embedded in resin, and thin-sectioned. Grids were incubated for 1 h with rabbit antibodies against lipophilic LPS isolated from *Mesorhizobium loti* NZP2213 grown under free-living conditions, and then with goat anti-rabbit IgG conjugated with 15 nm gold particles. The immunogold-labelling of bacteroids was analysed using a JEM 100C transmission electron microscope as described by Janczarek *et al*. (2001). Control specimens were nodule sections incubated with the gold-complexed secondary antibodies alone. Serum against the S-form LPS was produced in two New Zealand white rabbits according to the Yang and Lin (1998) protocol as described in Turska-Szewczuk *et al*. (2007).

SDS/polyacrylamide gel electrophoresis and immunoblotting. Rapid isolation of LPS from untreated or de-O-acetylated whole proteinase K-digested cells of the wild-type *M. loti* strain was performed according to the method described by Apicella *et al.* (1994).

LPS was analysed by tricine SDS/PAGE and visualised by silver staining according to a method described earlier (Turska-Szewczuk *et al*., 2007).

For immunochemical analysis, LPS separated by SDS/PAGE was transferred to Immobilon P (Millipore). Rabbit antibodies against LPS from the phenol phase of *M. loti* NZP2213 was raised according to the schedule described by Biosca *et al*. (1996). The primary antibodies were detected using alkaline phosphatase-conjugated goat anti-rabbit antibodies (Sigma). Blots were developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate toluidine (Sigma) for 5 to 15 min.

RESULTS

LPS of *Mesorhizobium loti* mutant 2213.1 does not function as a phage A1 receptor

The adsorption of phage to isolated receptor molecules is known to inactivate the phage particles. Therefore, in order to compare the receptor activity of *M. loti* wild-type and mutant LPSs, phage A1 particles were incubated with purified lipopolysaccharide preparations. S-type LPS of *M. loti* NZP2213 at a concentration of 1 or 10 μg/ml reduced the bactericidal activity of phage A1 particles to 0.1%. In contrast, no inactivation occurred after incubation with the LPS of the mutant strain 2213.1, which could indicate alterations in the LPS structure. To gain further insight into the nature of *M. loti* NZP2213 LPS as a phage A1 receptor, alkaline de-O-acetylation was performed. Treatment of S-type LPS or heat-killed whole bacterial cells of the wild-type strain with 0.5 M sodium methoxide allowed elimination of O-acetyl groups from position 2 of 6-deoxytalose residues and revealed that such an elimination was sufficient to completely impair the adsorption of phage A1 particles.

Spectroscopic analysis of the 2213.1 mutant O-polysaccharide

Bacterial cells of mutant 2213.1 were extracted with aq. 45% phenol and the S-form lipopolysaccharide recovered from the phenol phase was degraded with 1.5% acetic acid to give the precipitating lipid A and a degraded polysaccharide solution. The polysaccharide portion was subjected to fractionation by gel filtration chromatography on a Sephadex G-50 column, and the O-specific PS was eluted from the column in the void volume. Sugar analysis of the high-molecular-weight fraction, including establishing the absolute configurations of monosaccharides, showed the presence of mainly 2-*O*-methyl-6-deoxy-L-talose and 6-deoxy-L-talose (L-6dTal) in a molar ratio of 1.2 : 7, determined as their alditol acetates on GC-MS. In addition, small amounts of rhamnose, *N*-acetylquinovosamine and 3-deoxy-2-octulosonic acid (Kdo) were also detected. A GC-MS analysis of permethylated oligosacharide alditols derived from partially hydrolysed N-deacylated OPS indicated the presence of a trisaccharide which, based on MS fragmentation data, was identified as methylated 6-deoxyhexosyl-quinovosaminyl-3-deoxyoctonate alditol (not shown), which seems to have originated from the O-chain core linker.

An analysis of partially methylated alditol acetates derived from trideuteriomethylated O-specific PS of 2213.1 revealed the presence of 2,4-di-*O*-

Table 1. ^{1}H NMR and ^{13}C NMR chemical shifts (δ in ppm) of O-specific polysaccharide of *M. loti* mutant 2213.1

Sugar residue	H-1 C-1	H-2 C-2	H-3 C-3	H-4 C-4	H-5 C-5	H-6 C-6	OCH_3	CH_3CO
→3)-α-L-2–*O*-Ac-6dTal*p*^{A}-(1→	5.10 97.13	5.12 70.72	4.09 71.00	3.94 68.50	4.07 68.70	1.28 16.79		2.19 21.81
→3)-α-L-6dTal*p*^{B}-(1→	5.17 99.90	4.04 70.72	4.10 72.70	3.98 70.40	4.19 69.05	1.29 16.79		
→3)-α-L-2–*O*-Me-6dTal*p*^{C}-(1→	5.31 96.70	3.73 79.76	4.18 72.70	3.89 70.30	4.16 71.50	1.31 16.79	3.53 60.05	

CD_3-6-deoxytalose as the major component, while 2-*O*-methyl-4-*O*-CD_3-6-deoxytalose was detected only in smaller amounts. The trideuteriomethylation analysis thus proved that strain 2213.1 synthesized unbranched O-specific PS composed of 1,3-linked 6-deoxyhexose residues. These data also showed that all the sugar residues were 6-deoxytalopyranoses (6dTal*p*).

The ^{1}H and ^{13}C NMR spectra (Table 1) of the 2213.1 mutant O-polysaccharide contained signals of different intensities, thus showing a lack of strict regularity. The ^{13}C NMR spectrum (Table 1) showed, *inter alia*, signals for three anomeric carbons at δ 96.7, 97.13 and 99.9, CH_3-C groups (C-6 of 6dTal) at 16.79, two carbon atoms of an O-acetyl group (CH_3 at δ 21.81, CO at δ 174.47), and one O-methyl group (CH_3-O at δ 60.05). In the low-field region of the ^{1}H NMR spectrum of 2213.1 OPS (Table 1), out of four signals at δ 5.10, 5.17, 5.31, and 5.12, three were from anomeric protons and one, at 5.12 ppm, was assigned to a downfield-shifted signal of H-2 of 2-O-acetylated 6dTal by comparison with the corresponding signal from a nonacetylated sugar residue (δ 4.04). This assignment was confirmed by the HSQC spectrum (Fig. 1) which showed a correlation between a proton signal at δ 5.12 and the signal from an analogous carbon atom at δ 70.72. The high-field region of the ^{1}H NMR spectrum contained signals for an O-acetyl group (CH_3 at δ 2.19) and three doublets at δ 1.28-1.31 corresponding to methyl groups of 6-deoxy sugars (Table 1).

The ^{1}H and ^{13}C NMR spectra of the OPS of 2213.1 were assigned using 2D COSY, TOCSY, NOESY and H-detected ^{1}H, ^{13}C HSQC experiments (Tables 1 and 2). In the TOCSY spectrum, there were cross-peaks between H-1 and H-2,3,4 of 6dTal*p* and 2-O-methylated 6dTal*p* residues, but only H-1,H-2, H-2,H-3 and H-2,H-4 cross-peaks of 2-O-acetylated 6dTal*p*. The NOESY spectrum showed H-1,H-2, H-4,H-5, and H-4,H-6 correlations for all the sugar

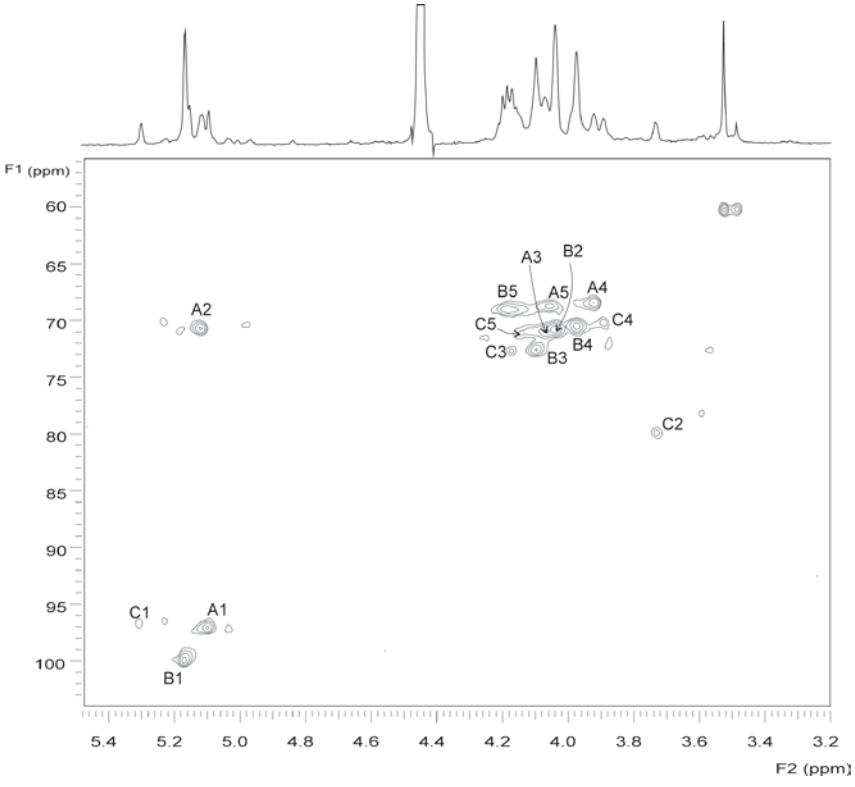


Figure 1. Partial ^{1}H, ^{13}C HSQC spectrum of O-specific polysaccharide of *M. loti* mutant 2213.1. The map shows peaks arising from carbon-proton pairs. A, B and C refer to 2-*O*-acetyl-6-deoxytalose, 6-deoxytalose, and 2-*O*-methyl-6-deoxytalose, respectively.

Table 2. Significant NOE signals observed for anomeric protons of O-specific PS of *M. loti* mutant 2213.1

Anomeric proton	NOE to proton			
Sugar residue	δ_H	δ_H	Residue	Atom
→3)-α-L-2–*O*-Ac-6dTal*p*^{A}–(1→	5.10	4.10	B	H-3[a]
→3)-α-L-6dTal*p*^{B}-(1→	5.17	4.09	A	H-3[a]
		4.10	B	H-3[a]
		4.18	C	H-3[a]
		3.89	C	H-4[c]
		4.04	B	H-2[b]
→3)-α-L-2–*O*-Me-6dTal*p*^{C}-(1→	5.31	4.10	B	H-3[a]
		3.98	B	H-4[c]
		3.73	C	H-2[b]

[a]Equatorial-axial coupling of neighboring protons across the glycosidic linkage; [b]Correlation of protons of the same sugar residue; [c]Minor cross-peaks between H-1 and H-4 protons indicate, at variance with methylation analysis data, closely spaced protons of neighbouring sugar residues.

residues. Other ^{1}H NMR signals of 6dTal*p* residues were assigned using correlations between the coupled protons in the COSY spectrum.

The $^1J_{C,\ H}$ coupling constant values (above 175 Hz) indicated that all the sugar residues had an α anomeric configuration. The α configuration of 6dTal*p* residues was also confirmed by the positions of the signals for H-5 (at δ 4.07–4.16 ppm) and C-5 (at δ 68.70–71.50 ppm), as was observed in the HSQC spectrum (Fig. 1), and by the intra-residual H-1,H-2 correlations for all 6-deoxyhexoses observed in the 2D NOESY experiment (Table 2).

The stereoconfigurations of 6-deoxytalose residues were identified according to the $^3J_{2,3}$, $^3J_{3,4}$ and $^3J_{4,5}$ coupling constant values of 3.5 Hz, 3.5 Hz, and less than 1 Hz, respectively, as expected for the *talo* configuration (Knirel *et al*., 1992; Russa *et al*., 1995b).

The ^{1}H NMR spectrum of 2213.1 OPS, showing comparatively low intensity of both the H-2 signal of 2-*O*-acetyl-6dTal*p* and the signal at δ 2.19 ppm corresponding to O-acetyl groups, indicated a significant decrease of O-acetylation of 6dTal*p* residues in the mutant O-polysaccharide relative to that of the wild-type strain where O-acetylation was complete (Russa *et al*., 1995b). A comparison of the integrated areas of anomeric protons of unsubstituted 6dTal (B) with those of 2-*O*-acetyl-6dTal (A) and 2-*O*-methyl-6dTal (C) showed that the O-specific PS of 2213.1 was only partially O-acetylated and O-methylated (about 25% and 12.5%, respectively).

The sequence and linkage analyses of the 2213.1 OPS were performed using sequential pre-irradiation of each of the anomeric protons in a 2D NOESY experiment. The NOESY spectrum demonstrated an inter-residue correlation (Table 2) between the following anomeric protons and the protons at the linkage carbons:

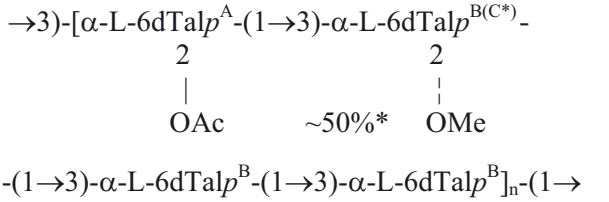
B H-1 and B H-3 at δ 5.17/4.10; B H-1 and C H-3 at δ 5.17/4.18; C H-1 and B H-3 at δ 5.31/4.10; B H-1 and A H-3 at δ 5.17/4.09; A H-1 and B H-3 at δ 5.10/4.10 ppm.

In addition to the correlations listed above, weak NOEs between some neighbouring protons were observed. The 1→3 position of the glycosidic linkages between 6dTal*p* residues as well as the substitution of the polysaccharide with O-acetyl and O-methyl groups made the mutant O-antigen poorly soluble (below 10 mg/ml) and caused weakening of some cross-peaks, as observed in 2D spectra. In accordance with the *talo* configuration of the anomeric centres, the presence of additional intra- and inter-residue responses for pre-irradiation of anomeric protons of H-2 and H-4 of the same or a neighbouring sugar residue is the expected result for α 1→3-linked 6-deoxytaloses (Table 2), where the anomeric protons are close to H-2 of the same residue and to the equatorial H-4 of the glycosylated sugar, as has been reported for the same glycosylation pattern of 6-deoxyhexoses (Knirel *et al*., 1992; Russa *et al*., 1995b).

After assignment of ^{13}C resonances from the HSQC data, the HMBC experiment shown in Fig. 2 provided long-range proton-carbon correlations across the glycosidic linkages, and the result supported the NOE data:

B H-1 and B C-3; B H-1 and C C-3; C H-1 and B C-3; B H-1 and A C-3; A H-1 and A C-3.

Taking into account the data obtained, it was concluded that the structure of the mutant 2213.1 O-polysaccharide should be postulated as follows:



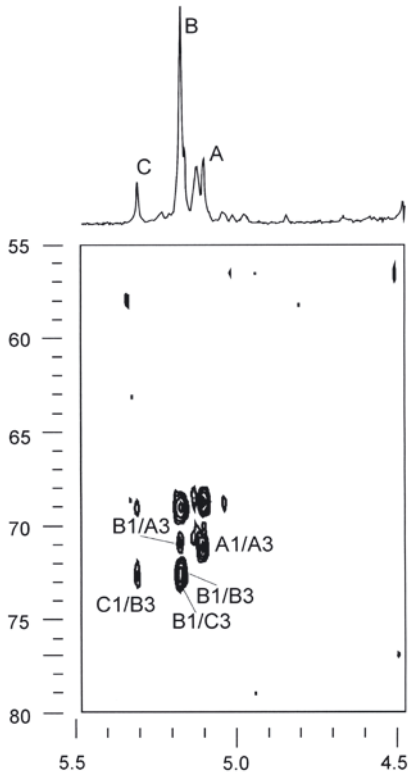


Figure 2. Part of 2D HMBC spectrum of O-specific polysaccharide of *M. loti* mutant 2213.1.
The map shows connectivities involving anomeric ^{1}H–^{13}C resonances. Arabic numerals before and after the oblique stroke refer to protons and carbons, respectively, in the sugar residues denoted by letters as shown in Table 1. Only transglycosidic correlations are marked.

Immunogold and Western immunoblot analyses

To assess whether the LPS epitopes present in the wild-type strain in free-living bacteria are also expressed in bacteroids, immunostaining was applied. The specificity of labelling was evidenced by the presence of gold particles only at the periphery of the endosymbionts. We can assume that these antibodies were directed against the O-antigen portion of LPS. The micrographs showed that the LPS epitope(s) were differently displayed on the surface of the wild-type and mutant bacteroids. While the cell surfaces of the parental bacteroids were relatively densely immunostained, much less labelling was observed on the mutant cell surfaces (Fig. 3a, b).

Western immunoblot data revealed that polyclonal antibodies against S-form LPS of NZP2213 reacted only with higher-molecular-weight bands of both homologous (wild-type) and mutant LPSs (Fig. 4b, lanes 2 and 6). On the other hand, de-O-acetylated S-form LPS of the wild-type strain as well as the parental cells subjected to alkaline methanolysis showed almost no reactivity with homologous serum (lanes 8 and 10), in contrast to a positive reaction with the low mobility band on LPS profiles from untreated cells (lane 9). The latter results as well as the lower reactivity with antibodies of partially de-O-acetylated S-LPS of the wild-type strain (lane 7), indicate that 2-O-acetylation of 6dTal residues is critical for LPS antigenic properties. It is worth mentioning that serum against R-type LPS isolated from the water phase interacted only with a faster-running band lacking the O-polysaccharide portion (not shown).

DISCUSSION

In this report, we describe a structural investigation of the OPS of the transposon mutant 2213.1 derived from *M. loti* NZP2213 (Turska-Szewczuk *et al*., 2007). A comparative analysis of ^{1}H NMR spectra showed differences between the mutant and

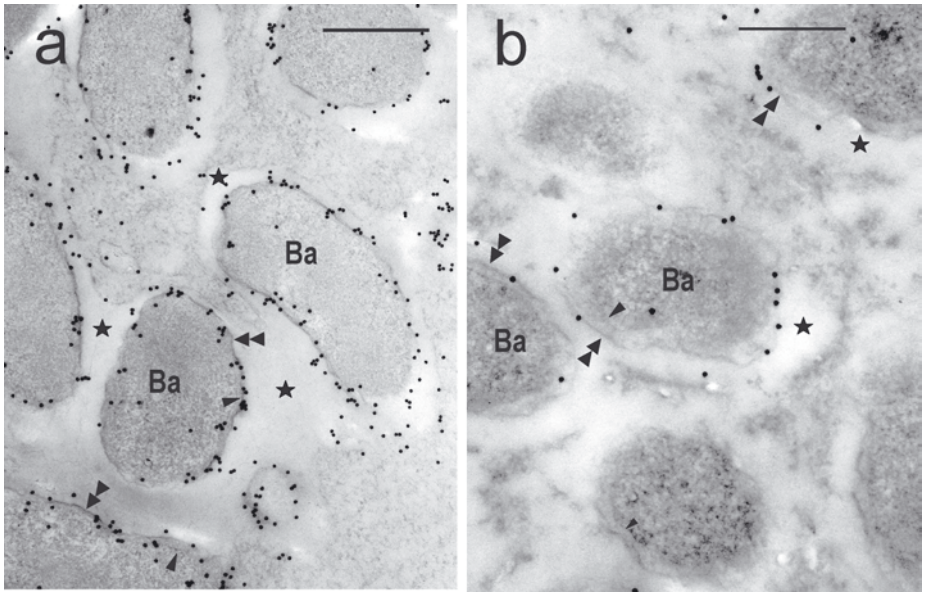


Figure 3. Immunogold localization of LPS on bacteroid cell surface in *Lotus corniculatus* nodules induced by *M. loti* wild-type and mutant 2213.1 strains.
Differently displayed gold particles on the surface of the wild-type (a) and mutant bacteroids (b). Outer membrane of bacteroid = double arrowheads, inner membrane of bacteroid = arrowheads, and peribacteroid space = asterisks. Abbreviation: Ba = bacteroids. Bars: 0.5 μm (a); 0.4 μm (b).

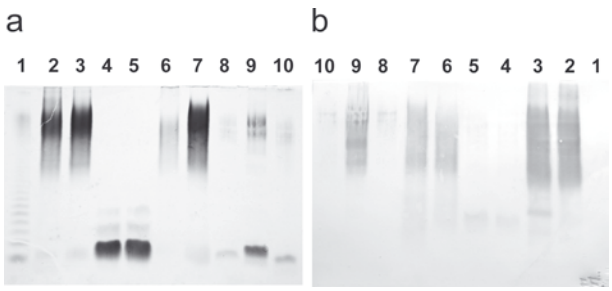


Figure 4. Western immunoblot of LPSs detected with antibodies against S-type LPS of the wild-type *M. loti* strain.
Immunoblot (b): 1, LPS of *S. enterica* sv. Typhimurium; 2, and 3, lipophilic S-type LPS of NZP2213; 4, water soluble R-type LPS of mutant 2213.1; 5, R-type LPS of NZP2213; 6, S-type LPS of the mutant strain; 7, partially de-O-acetylated S-type LPS of the wild-type strain; 8, completely de-O-acetylated S-type LPS of the wild-type strain; 9, LPS from proteinase K-digested whole bacterial cells of the wild-type strain; 10, LPS from proteinase K-digested, alkaline-treated *M. loti* NZP2213 bacterial cells. Approximately 5 μg of LPS was loaded per lane except 3 μg for lane 2. SDS/PAGE (a) was silver-stained after being electroblotted.

wild-type O-specific PS structures. In addition to decreased H-1 and H-2 signals of 2-O-acetylated 6-deoxytalose residue in the mutant OPS there were observed two additional anomeric proton signals of non-substituted and 2-O-methylated 6dTal residues. These data indicate that while the parental O-chain represents a homopolymer composed of α 1→ 3-linked 2-*O*-acetyl-6dTal*p* (Russa *et al*., 1995b), the mutant O-chain is composed of tetrasaccharide repeating units consisting of non-substituted as well as O-acetylated or O-methylated 6dTal*p* residues joined by α 1→3 linkages.

The O-antigen polysaccharide of *Rhizobium etli* CE3 LPS is linked to the inner core *via* the outer core oligosaccharide composed of mannose, fucose, *N*-acetylquinovosamine and Kdo residues (Forsberg *et al*., 2003). The small amounts of rhamnose, Kdo and *N*-acetylquinovosamine detected both in the 2213.1 mutant and wild-type O-polysaccharides (Turska-Szewczuk *et al*., 2007) indicate that these sugar residues could form a glycosyl sequence, presumably deoxyhexosyl-(1→ 3)-quinovosaminyl-(1→4)-3-deoxyoctonate, involved in joining the OPS to the Kdo-terminated core region of *M. loti* LPS. A similarly composed oligosaccharidic sequence was detected in the LPS of *R. leguminosarum* bv. *trifolii* strain 24 (Russa *et al*., 1996). These data allowed us to hypothesize that O-specific polysaccharides of both the homopolymer of *M. loti* and the heteropolymers of *R. etli* CE3 and *R. leguminosarum* bv. *trifolii* 24 have a similar oligosaccharide attachment region, although the route of synthesis of these polymers on the undecaprenyl phosphate carrier might be quite different.

Our previous comparative results of SDS/PAGE electrophoresis and analysis of sugar composition indicated that LPS of the mutant strain possessed only half of the amount of hydrophobic O-polysaccharide as compared to the parental LPS.

This substantial decrease of O-chain substitution seems to be correlated with 2-*O*-acetyl-6-deoxytalose deficiency (about 50%) (Turska-Szewczuk *et al*., 2007). The scarcity of the main O-antigen sugar component could be a consequence of transposon insertion affecting a locus in the mutant genome coding for a specific glycosyltransferase (Turska-Szewczuk *et al*., 2007). One can assume that the mutant-elicited chronic infection, characterised by the formation of distorted infection threads and premature senescence of symbiosomes, could result from incompatible interactions between the plant cell and bacterial cells with the unfavourably altered LPS (Turska-Szewczuk *et al*., 2007).

The observed resistance of the mutant strain to phage A1 lysis, requiring intact LPS as a receptor (Turska-Szewczuk & Russa, 2000), suggested that the mutant LPS might be altered. Those data were supported by the inability of the mutant LPS to inactivate phage A1, while the lipophilic S-type LPS of *M. loti* NZP2213 strain reduced the bactericidal activity of phage A1 particles to 0.1%. We assumed that the failure of the mutant O-polysaccharide to serve as a receptor for phage A1 adsorption was caused by an alteration in O-acetylation and O-methylation of 6-deoxytalose residues. These data became a premise for testing whether O-acetylation of 6-deoxytalan is essential for wild-type LPS to be recognized as a phage A1 receptor. Our experiment clearly showed that removal of O-acetyl groups from the OPS was sufficient to prevent phage attachment to host cells.

Some surface polysaccharides are considered to be signalling molecules in the plant-microbe dialogue (Soto *et al*., 2006). It is still not known which structural determinants of *M. loti* LPS are required to initiate and sustain effective symbiosis with *L. corniculatus*. An analysis of phenotypes of LPS mutants has led to the conclusion that nearly-normal amount of the O-antigen portion of LPS is required for it to trigger a symbiotic response (Noel *et al*., 2000).

The results of the immunogold assay indicated a diminished amount of the O-antigenic determinant in the mutant LPS. The significant reduction of the number of gold particles seen on the mutant bacteroid cell surface could be caused by both the diminished content of the O-antigenic determinant in the mutant LPS and modifications of structural epitopes.

Results reported by Kannenberg & Carlson (2001) revealed that relatively modest changes, such

as alterations in the position of O-acetylation or O-methylation of sugar residues, resulted in significant changes in antibody reactivity of *R. leguminosarum* LPS.

Our Western immunoblot analysis with polyclonal serum anti S-type LPS of the wild-type strain showed that both the parental and mutant LPSs have similar O-antigens.

Lam *et al.* (1992) reported that substituents such as O-acetyl or O-methyl groups in the O-antigen chain are often important components of the antigenic determinant.

To check if this was also the case for *M. loti* LPS, the de-O-acetylation procedure was performed and its effect on antibody reactivity examined. Western immunoblot revealed that de-O-acetylated LPS or alkaline-treated wild-type cells had lost the reactivity with homologous anti S-form LPS serum. This may indicate that this chemical substituent is a crucial antigenic determinant.

Studies performed by Brett *et al*. (2003) on *Burkholderia pseudomallei* and *B. thailandensis* O-antigens have revealed that these species express similar O-polysaccharides, in which 6-deoxy-α-L-talopyranosyl residues are variably substituted with acetyl groups at the O-2 or O-4 positions. Pairwise immunochemical comparisons showed that O-acetylation of L-6dTal*p* at the O-2 position is critical for recognition by monoclonal antibodies, and it is highly probable that such modification serves as a structural epitope (Brett *et al*., 2003).

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