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Correlation between the occurrence of plasmid pUCS202 and lipopolysaccharide alterations in *Rhizobium*

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1. INTRODUCTION

The process of *Rhizobium*-legume symbiosis requires the participation of chromosomal genes of both partners and involves several stages such as the adsorption of bacteria onto the root hairs, penetration into the cortex, and development of nodules [1]. Recent results of physical and genetical studies of *Rhizobium* have shown that also extrachromosomal genes located on plasmids of different sizes can determine nodulation ability [2] as well as symbiotic properties [3,4]. It has been demonstrated that globular proteins (lectins) present on the surface of host roots are able to bind to the homologous symbiont cells [5,6]. Receptor sites for lectins have been found in the capsular polysaccharides of *R. trifolii* [7], in glucan-type polysaccharide of *R. leguminosarum* [8], in lipopolysaccharides (LPS) of *R. japonicum, R. leguminosarum,* and *R. phaseoli* [9] or in both these polymers of *R. japonicum* [10]. It is also interesting to note that rhizobial LPS have been able to act as kinetins and stimulate the proliferation of cortex tissue in pea root explants [11].

No direct correlation between LPS structure and nodulation specificity could be seen by comparing LPS isolated from different rhizobial strains from one different species [12–14].

The aim of this study was to determine the composition of LPS isolated from genetically characterized and closely related nodulating and non-nodulating strains of *R. trifolii* [15]. The results point to a correlation between the occurrence of the plasmid pUCS202 and LPS alterations in *R. trifolii.*

2. MATERIALS AND METHODS

In this study we used nodulating and non-nodulating strains of *R. trifolii* (Table 1). The bacteria were grown at 28°C in aerated liquid medium "79" supplemented with Difco casamino acids (5 g/l) [16].

Lipopolysaccharides were extracted by the phenol–water method [17] and purified by repeated ultracentrifugation at 105 000 × *g*. Intact LPS preparations were filtered through a column of Ultrogel AcA 22 (50 × 1.5 cm) equilibrated against 47 mM pyridinum–acetate buffer, pH 4.26. Disaggregated LPS preparations obtained by Triton X-100 treatment were fractionated on an Ultrogel AcA 22 column using pyridinum–acetate buffer supplemented with 0.02% Triton X-100 and 1 mM Na_2EDTA. Degraded polysaccharides obtained by mild acid hydrolises of LPS were fractionated on a Sephadex G-50 fine column (80 × 0.9 cm) using pyridinum acetate buffer. The carbohydrate content was estimated by the anthrone procedure as glucose equivalents. Oxidized lipopolysaccharides were obtained by treatment with 0.025 M $NaJO_4$ (4°C, 4 days) [18].

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Table 1

Relevant characteristics of *R. trifolii* strains

Strain	Nodulation	Plasmids [a] (in megadaltons)	Sensitivity to phage 3H [b]	Origin
24K	–	100, 15	–	Non-nodulating mutant of *R. trifolii* strain 24 [15]
24K56	+	100, 40/pUCS202/	+	Derived from 24K by conjugal transfer of the hybrid plasmid pUCS202 (ca. 40 megadaltons) which is composed of R6845 and a DNA fragment containing Nod (genes) [15].
24KOA	–	100	–	24KOA was obtained from 24K56 by elimination of pUCS202 using acridine orange [15].

[a] Molecular of plasmids were determined electrophoretically [21,22].
[b] Phage sensitivity was tested by dropping phage 3H dilutions on plates spread with tested rhizobia [24].

Sugars in LPS hydrolysates were converted to alditol acetates as previously described [19,20]. The alditol acetates were analyzed on a 0.3×180 cm column containing 3% ECNSS-M on Gas Chrom Q (100–120 mesh) at 190°C in a stream of nitrogen (30 ml/min) on a Varian Aerograph fitted with a Hewlett-Packard electronic integrator. Identification was done on the basis of retention times and, for some samples, by mass spectra taken with the Finnigan quadrupol 3200 mass-spectrometer. Amino sugars and their phosphate derivatives obtained after LPS hydrolysis (4 N HCl, 8 h, 100°C) were estimated on a Durrum (D-500) automatic amino acid analyzer.

Plasmid DNA was isolated from exponentially growing cultures and analyzed on horizontal agarose gels [21,22].

3. RESULTS

The analysis of LPS preparations purified by repeated ultracentrifugation showed that all the preparations contained neutral sugars, amino sugars, 2-keto-3-deoxyoctonate, Lipid A and trace amounts of amino acids: aspartic acid, threonine, serine, glutamic acid, alanine, valine, methionine, leucine, phenylalanine and arginine. The ratios of glucosamine phosphate to glucosamine were higher in the hydrolyzed LPS of the nonnodulating strains than in the corresponding material of the nodulating one. An unkown component producing a peak at the position of glyceryl-*S*-cysteine on amino acid analysis profiles was detected only in the LPS of the nodulating strain (Table 2).

Quinovosamine was found only in the nodulating strain carrying the plasmid pUCS202. The non-nodulating *R. trifolii* 24K which did not have quinovosamine, acquired after the introduction of pUCS202 (strain 24K56), simultaneously the capability for nodulation and quinovosamine synthesis. These effects were reverted by elimination of pUCS202 strain (24 KOA) with acridine orange.

The intact LPS preparations were homogeneous when filtered through an Ultrogel AcA 22 column and nearly 95% of the material was eluted as particles of $M_r = 1\text{–}2 \cdot 10^6$. The neutral sugar composition of this material was the same as in LPS preparations purified by ultracentrifugation. Glucose and rhamnose were the most abundant constituents of the LPS of the nodulating strain whereas the non-nodulating strains contained mainly fucose, rhamnose and mannose (Table 2). The differences in composition between LPS 24K56 on the one hand and of the 24KOA and 24K on the other were present also in periodate-oxidized preparations (Table 3).

Because of the high glucose content of the LPS of 24K56 we additionally fractionated it on Ultrogel AcA 22 column in the presence of disaggregating agent Triton X-100 and Na_2EDTA. Under these conditions we obtained two peaks: the

Table 2

Chemical composition of *R. trifolii* lipopolysaccharides

Component	Nodulating		Non-nodulating	
	24K56 [a]	24K56 [b]	24K [a]	24KOA [a]
Rhamnose	41.3	43.4	26.7	32.1
Fucose	–	–	39.6	36.3
Xylose	4.9	–	–	–
Mannose	9.0	14.9	19.6	18.8
Galactose	4.6	11.1	5.1	6.4
Glucose	30.9	13.7	2.1	1.9
Heptose	traces	traces	traces	traces
Unknown	1.1	–	–	–
Glucosamine [c]	198.4	N.d.	114.0	166.0
Quinovosamine [c]	44.0	N.d.	–	6.0
Glucosamine 6-phosphate [c]	35.2	N.d.	42.6	66.4
Unknown [d]	+	N.d.	–	–

Values for neutral sugars are given as mol %.

[a] LPS purified by repeated ultracentrifugation at 105000×*g* for 3 h.

[b] LPS purified on Ultrogel AcA22 column in the presence of Triton X-100 (0.02%) and 1 mM Na_2EDTA.

[c] Amino sugar contents are expressed in nmol/mg of LPS determined by amino acid autoanalysis.

[d] Unknown component forming peak at the position of glyceryl-*S*-cysteine on amino acid analysis profiles.

N.d., not determined.

first, of $M_r = 1\text{–}2 \cdot 10^6$, contained almost exclusively glucose whereas the second, of $M_r = 0.5\text{–}1.0 \cdot 10^5$ contained LPS composed mainly of rhamnose, mannose, glucose and galactose in a ratio of 4:1:1:1 (Table 2).

The degraded polysaccharide preparations isolated from the LPS were of predominantly high M_r (approx. $1.0 \cdot 10^4$) independently of the strain used, although they differed in chemical composition. The polysaccharide of 24K56 had a high content of rhamnose, glucose and mannose whereas those of 24KOA and 24K contained mainly rhamnose, fucose and mannose.

The differences in LPS composition between the nodulating strain 24K56 and the two non-nodulating strains 24KOA and 24K also correlated with their sensitivity to the phage 3H (Table 1).

Table 3

Sugar components in lipopolysaccharides oxidized with periodate (mol%)

Sugar	Nodulating	Non-nodulating	
	24K56	24K	24KOA
Glycerol	8.2	12.0	13.7
Erythritol	10.3	–	0.8
Threitol	27.1	–	–
Rhamnose	23.9	17.0	17.6
Fucose	–	50.7	48.2
Xylose	6.4	6.9	8.4
Mannose	5.8	4.2	5.4
Galactose	2.1	–	–
Glucose	6.4	0.4	0.3

4. DISCUSSION

R. trifolii lipopolysaccharides consist of lipid A ketosidically linked to the polysaccharide chain containing hexoses, deoxyhexoses, *O*-methyldeoxyhexoses, amino sugars and uronic acids [12,13]. Heptoses and methylheptoses are rarely present [20]. Glucose, rhamnose and sometimes fucose occur at higher concentrations than other sugar components.

The present study revealed that fucose, glucose

and quinovosamine were constituents distinguishing the LPS of nodulating and non-nodulating strains. These differences did not result from S → R mutation as was demonstrated by analyzing high M_r fractions of the degraded polysaccharides.

The *R. trifolii* strain harboring pUCS202 was able to synthetise glucan of an M_r of approx. $1.0 \cdot 10^6$. We have not yet determined the structure and the possible role of this polymer in the nodulation process. It is interesting to note that the non-nodulating strain 24K, upon the acquirement of pUCS202 (strain 24K56) showed alterations in LPS composition which were reversed by elimination of the plasmid pUCS202. This was confirmed by periodate oxidation of LPS. According to this analysis the polysaccharide parts of 24K and 24KOA were similar.

Not only the neutral sugars but also the amino sugar composition were altered by the presence of pUCS202. Amounts of quinovosamine found in the LPS of the nodulating strain were larger than in the non-nodulating ones. Recently it has been found that trifollin-binding receptors on the surface of *R. trifolii* appeared simultaneously with quinovosamine at the early stationary phase of growth [5]. The reactivity to lectins together with presented differences in sugar composition of LPS from nodulating and non-nodulating strains support the hypothesis that lipopolysaccharides can participate in specific recognition of rhizobia by host plants. However, the mechanism of this interaction is still unknown and strains nodulating the same legumes are frequently different in O-antigenic specificity and phage pattern [12,13,16,24]. The analysis of the molecular basis for this symbiont–host selectivity should be completed by structural studies. The mechanism of the plasmid-mediated alterations of LPS remains still unknown suffice it to point at several other instances of plasmid-determined cell envelope changes [25–27].

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