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Characterization of two lipopolysaccharide types isolated from *Rhizobium galegae**

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Lipopolysaccharides (LPS) of *Rhizobium galegae*, a symbiotically nitrogen-fixing species of root-nodule bacteria, were isolated by the phenol-water method from strain HAMBI 1461, the LPS of which resembled enterobacterial smooth type LPS, and from strains HAMBI 1174 and HAMBI 1208, the LPSs of which resembled rough type LPS. The results of PAGE analysis of LPSs, Bio-Gel P2 gel filtration of polysaccharide fractions and the presence of deoxysugars and 4-*O*-methyl-deoxysugar both in the rough and smooth LPSs suggested that rough LPS contained a short O-antigenic polysaccharide for which we propose the name short O-chain LPS. Accordingly, the smooth LPS is called long O-chain LPS. Despite of the differences in the structure of LPS of *R. galegae*, all strains were equally effective in nodulating their hosts. The short O-chain LPS of *R. galegae* showed many features similar to those of phylogenetically related agrobacteria.

The outer membrane of wild-type Gram-negative bacteria contains a mixture of complete and incomplete lipopolysaccharide (LPS) molecules. Complete LPS molecules consist of lipid A, core oligosaccharide (core) and O-antigenic polysaccharide (O-chain). Incomplete molecules contain lipid A and core only. In many cases the same bacteria have O-chains of different length. Only certain groups of bacteria inhabiting mucosal surfaces such as the respiratory and genito-urinary tracts (e.g. *Neisseria* sp.) possess LPSs which naturally lack the O-chain [1]. Bacteria of the genera *Azorhizobium, Rhizobium* and *Bradyrhizobium* are soil organisms belonging to the α-subgroup of *Proteobacteria* which are able to form a nitrogen-fixing symbiotic relationship with leguminous plants. On PAGE profiles the rhizobial LPS can be seen as two main banding regions. The region of lower mobility is called LPS I and corresponds to complete LPS mole-

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Abbreviations: Doc, sodium deoxycholate; LPS, lipopolysaccharides.

cules, whereas the region of higher mobility called LPS II contains incomplete molecules lacking the O-chain. LPS I has been shown to be important for the process of infection by some rhizobial species; lipopolysaccharide mutants of *R. leguminosarum, R. etli* or *B. japonicum* which lack O-chains cannot induce nitrogen fixing nodules [2–4].

The LPSs of *R. galegae* strains, which nodulate *Galegae orientalis* (goat's rue) and *G. officinalis* (French lilac), seemed to differ from those of other rhizobia. SDS and sodium deoxycholate/PAGE analysis of cells treated with proteinase K showed that several *R. galegae* strains had LPS profiles resembling the rough LPS type of enteric bacteria [5, 6]. Apart from these strains two *R. galegae* isolates having smooth type LPS were detected ([5] and G. Radeva, personal communication). The aim of our study was to characterise the LPS types, and to clarify, whether the LPSs of rough *R. galegae* strains really lack O-antigenic polysaccharide chains.

MATERIALS AND METHODS

Bacterial cultures. *R. galegae* strains HAMBI 1174 (rough LPS), HAMBI 1208 (rough LPS) and HAMBI 1461 (smooth LPS) [5, 7] were used in this study. The strains were maintained in the culture collection of the Department of Applied Chemistry and Microbiology, University of Helsinki (HAMBI). For the isolation of LPS the bacteria were grown to stationary phase in aerated broth [5] at 22°C. The growth medium of strains 1174 and 1208 contained 0.2 mg ml^{-1} streptomycin.

Isolation of LPS. Bacterial pellets were washed once with 0.5 M NaCl and twice with sterile water. The LPSs were isolated with hot 45% phenol as described by Johnson & Perry [8]. Both the water and phenol layers were dialysed against tap water and then against deionized water, digested with proteinase K (0.1 mg ml^{-1}) at 37°C for 2 h, dialysed and freeze dried. The materials from the water phases of strains 1174 and 1461 were pelleted by ultracentrifugation (120000 × ***g***, 4°C, 4 h) and freeze dried. The material from the phenol phase of strain 1208 was suspended in water according to Carrion *et al.* [9].

LPS purification by chromatography on Sepharose 4B. LPS preparations were fractionated by Sepharose 4B chromatography with triethylamine/EDTA buffer, pH 7.0 [10]. The eluted fractions were assayed for 2-keto-3-deoxyoctonate according to Karkhanis *et al.* [11] and hexoses according to Spiro [12].

Mild acid hydrolysis and Bio-Gel P2 chromatography. The polysaccharide part of the LPS (purified by Sepharose 4B filtration) was separated from lipid A by mild acid hydrolysis [13]. After hydrolysis the freeze dried supernatant was applied to a Bio-Gel P2 (Bio-Rad Laboratories) column (1.2 m × 1 cm) using 1% (v/v) acetic acid as an eluent [14]. The eluted fractions were assayed for the presence of uronic acids [15] and hexoses [12].

Analytical methods. Fatty acids were determined as their methyl esters after methanolysis of the LPS samples with heptadecanoic acid added as the internal standard [16]. Analysis of LPS samples for carbohydrates was carried out after mild acid hydrolysis with 50 mM trifluoroacetic acid (TFA) at 100°C for 1 h and reduction with NaB^2H_4. The samples were then subjected to methanolysis, carboxy-reduction (NaB^2H_4), N-acetylation, acid hydrolysis (1 M TFA, 120°C for 2 h) and acetylation as described by Russa *et al.* [17]. The absolute configuration of sugars were determined by comparison of retention times of trimethylsilylated (–)-2-butyl glycosides prepared from LPS with those of authentic standards according to Gerwig *et al.* [18].

Gas chromatography-mass spectrometry of LPS compounds. The products obtained were analysed by GC-MS using a Hewlett-Packard 5890A mass spectrometer equipped with a capillary: a) DB 1701 fused silica column (25 m × 0.25 mm) for alditol acetates with the temperature program starting at 180°C for 10 min followed by a temperature increase of 10°C min^{-1} to 220°C which was maintained for 25 min; b) DB 5 (25 m × 0.2 mm) was used for trimethylsilyl (–)-2-butylglycosides at 150°C with a temperature increase of 2°C min^{-1} up to 300°C,

and for fatty acid methyl esters at 180°C for 5 min with subsequent temperature increase of 5°C min^{-1} up to 300°C.

PAGE. Discontinuous slab gel electrophoresis was performed with SDS [19] or sodium deoxycholate (Doc) [20] using a 4% (w/v) stacking gel and a 16% separating gel. Gels were silver stained [21] or Alcian Blue/silver stained [22].

RESULTS AND DISCUSSION

LPSs from the *R. galegae* strains 1174 and 1461 were derived during hot phenol-water extraction from the water phase whereas LPS from strain 1208 was dissolved mainly in the phenol phase with traces only remaining in the water layer. When the isolated LPSs were subjected to SDS- and Doc/PAGE and silver-stained, the smooth LPS of strain 1461 displayed a ladder-like pattern (Fig. 1a, 1b), which clearly indicated the presence of both LPS I with long O-antigenic polysaccharides of different length, and LPS II. Krauss *et al.* [20] showed that sodium deoxycholate had a higher dissociating power and separated LPS aggregates better than SDS did. This might explain, why in SDS/PAGE the LPS of strain 1174 gave two regions (LPS I and LPS II) very close to each other (Fig. 1b) whereas in Doc/PAGE only one broad band was visible (Fig. 1a). In SDS/PAGE LPS I and LPS II regions were distinguishable, because some LPS molecules formed aggregates. On the other hand Doc/PAGE could not separate LPS I and LPS II regions from each other because there were no LPS aggregates left, and the cores with O-chains and the cores without O-chains were detected as one

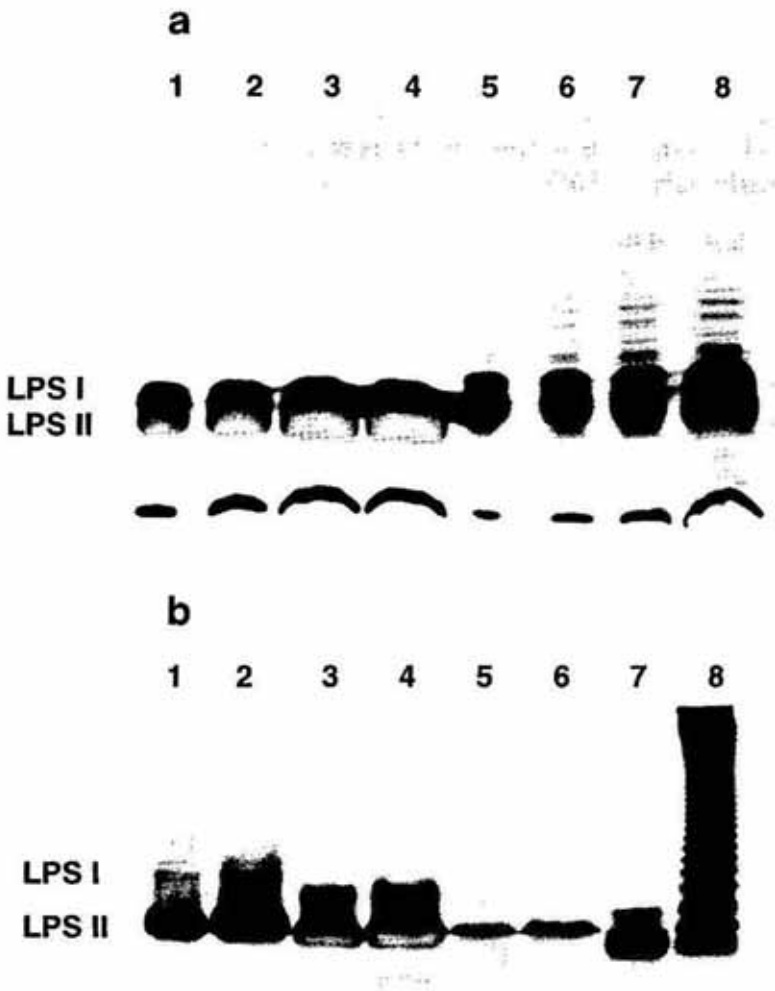

Figure 1. (a) 16% (w/v) Doc/PAGE and (b) 16% (w/v) SDS/PAGE analysis of LPS from *Rhizobium galegae* strains.

(a) Lanes 1 through 4, HAMBI 1174 LPS; 1.25 µg, 2.5 µg, 5 µg and 7.5 µg, respectively. Lanes 5 through 8, HAMBI 1461 LPS; 1.25 µg, 2.5 µg, 5.0 µg and 7.5 µg, respectively. (b) Lanes 1 and 2, HAMBI 1461 LPS; 2.5 µg and 5.0 µg. Lanes 3 and 4, HAMBI 1174 LPS; 2.5 µg and 5.0 µg. Lanes 5 and 6, HAMBI 1208 LPS; 2.5 µg and 5.0 µg. Lane 7, 1.8 µg of Ra-LPS from *Salmonella typhimurium* TV119. Lane 8, 3.6 µg S-LPS from wild type *S. typhimurium*. HAMBI 1174 and HAMBI 1461 LPSs were derived from water phases whereas HAMBI 1208 was obtained from phenol phase.

broad band. LPS of strain 1208 from the phenolic fraction was detected in SDS- (not shown) and Doc/PAGE as one narrow band in the LPS II region (Fig. 1b). When PAGE patterns of LPSs (Sepharose purified, not shown) were stained with Alcian Blue/silver [22] their electrophoretic profiles did not reveal any additional bands compared to those of the preparations directly silver stained. In the study of Reuhs *et al.* [23] the silver staining technique of Tsai & Frasch [21] stained LPS only, whereas the Alcian Blue/silver technique stained both the LPS and acidic polysaccharides. Therefore, it was concluded that the purified LPS preparations were free of acidic polysaccharides.

The fatty acid composition of the water soluble LPS of the strains 1174 and 1461 were very similar and characterised by a high proportion of hydroxy fatty acids. Phenol soluble LPS of strain 1208 had nearly equal amounts of hydroxy and nonhydroxy fatty acids (Table 1). The presence in isolated lipid A of octadecenoic (18:1), *cis*-11,12-methyleneoctadecanoic (19:0 cyc) and 11-methoxynonadecanoic acid (11-OMe-19:0) in the rough 1208 LPS was confirmed. Although these octadecenoic and methyleneoctadecanoic residues were found to be integral components of lipid A of *Rhodospirillum salinarum* [24] and *R. tropici* [25] we cannot exclude that they were derived from contamination of phenol soluble LPS by other cellular lipids. 27-Hydroxyoctacosanoic acid (27-OH-28:0), which was detected in all three *R. galegae* strains, is characteristic of all families (including *Rhizobiaceae*) belonging to the α-2 subdivision of the class *Proteobacteria* [26].

The neutral, acidic and amino sugars identified in the three strains of *R. galegae* are listed in Table 2. Fucose, galactose, glucose and mannose were the major neutral sugars present in the LPS of all three strains. Otherwise the neutral sugar composition varied between the strains. 3-*O*-Methylhexose was present exclusively in the 1461 LPS, whereas 4-*O*-methyl-6-deoxyhexose was a characteristic constituent of the 1174 LPS. The total content of neutral sugars was 2 to 3 times higher in the smooth LPS compared to that in the rough LPSs. The ratio of galacturonic acid (one of the core compounds of rhizobial LPS) to galactose was 3:1 and 3:2 in the

Table 1. Fatty acid composition of LPSs isolated from *R. galegae* strains.

Values are given in μg mg^{-1} of LPS.

Fatty acid	HAMBI 1461	HAMBI 1174	HAMBI 1208
14:0	3.7	3.6	0.5
16:0	3.9	5.4	12.3
18:1	12.1	8.3	63.3
18:0	3.9	2.9	0.5
19:0 cyc	0.5	1.6	18.3
11-methoxy-19:0	2.0	4.2	30.7
3-OH-14:0	35.3	50.7	20.3
3-OH-16:0	24.1	36.2	20.5
3-OH-18:1	23.9	47.8	19.3
3-OH-18:0	5.2	5.6	18.5
25-OH-26:0	0.5	3.9	–
27-OH-28:0	47.5	69.3	40.0

rough 1174 LPS and in the rough 1208 LPS, respectively, but 1:1 in the smooth 1461 LPS. The presence of 2-keto-3-deoxyoctonate in all LPSs was confirmed by GC-MS analysis of the peak identified as its 3-deoxy-1,1,2-trideuterio-octitol heptacetate. In alditol samples of the 1174 and 1461 LPSs another acidic compound which contained five deuterium atoms (1,1,2,7,7-d_5) was traced. The retention times and mass spectra of the 3-deoxyheptitol hexacetates were identical with those previously described for the derivative of 3-deoxy-*lyxo*-2-heptulosaric acid in the LPS of *R. leguminosarum* bv. *trifolii* [17]. 2-Keto-3-deoxyoctonate and D-glucosamine content was significantly higher in the LPSs of the rough strains than in that of the smooth one.

The separation of degraded polysaccharides on Bio-Gel P2 (not shown) revealed that mild acid hydrolysis produced two peaks corresponding to oligosaccharide and monosaccharide fractions eluted at the same volumes for all *R. galegae* strains. An additional polysaccharide fraction eluted at V_0 as the main peak was observed on the profile of the smooth strain 1461. These results are in agreement with PAGE analyses data suggesting that the cores in LPSs from strains 1174 and 1208 could be substituted with short O-chains and the core in LPS from strain 1461, with a long O-chain. This was also supported by the presence of deoxysugars and methylated deoxysugars in the LPSs from *R. galegae* strains examined, because these sugars have been reported as the compounds of O-chain region of rhizobial LPSs [2, 10]. Some strains of *Pseudomonas* and *Campylobacter* sp. had a rough appearance in PAGE because the O-chains were not stained by silver, probably because of their resistance to periodate oxidation [1]. In the case of *R. galegae* this was not likely, because immunoblotting of homologous LPS with antiserum against HAMBI 540 (parental strain of 1174) confirmed the rough appearance of 1174 [5].

It is mentioning that, despite differences in length of O-chains, all *R. galegae* strains were equally effective in nodulating their

Table 2. A relative molar ratio of sugars in lipopolysaccharides from *R. galegae*.

The retention times were measured on capillary DB-1701 column (25 m × 0.25 mm).

Retention time (min)	Component	HAMBI 1461	HAMBI 1174	HAMBI 1208
10.99	4-*O*-methyl-6-deoxyhexose	–	3.5	–
11.23	L-rhamnose	1.6	traces	2.0
11.50	L-fucose	5.3	10.8	2.9
16.25	not identified	traces	2.0	1.9
16.49	3-*O*-methylhexose	5.3	traces	–
16.61	D-mannose	9.7	traces	7.0
17.13	D-galactose*	13.0	22.1	20.1
17.13	D-galacturonic acid*	4.4	22.1	13.4
17.56	D-glucose	42.6	16.2	33.0
18.35	quinovosamine	1.8	3.1	–
20.45	3-deoxy-2-heptulosaric acid	0.5	1.4	–
27.02	3-deoxy-2-octulosonic acid	4.5	7.2	11.5
27.92	D-glucosamine	3.4	8.1	8.7

*The content of galacturonic acid and galactose was estimated by contribution of peak areas for ion fragment at 291 m/z and 289 m/z, respectively, in carboxy-reduced samples.

hosts [5]. Consequently, the length of O-chain was not crucial for the infection and nodule development [27].

Since the rough LPS of *R. galegae* is not really rough but possesses very short O-antigenic polysaccharide chains, we propose to call this type of LPS short O-chain LPS. Accordingly, the smooth rhizobial LPS is further called long O-chain LPS.

Phylogenetic analysis of rhizobia and agrobacteria based on 16S rRNA gene sequences showed that *R. galegae* was more related to *Agrobacterium tumefaciens, A. rubi* and *A. vitis* than to other fast growing rhizobia [28]. LPS data for *A. vitis* and *A. rubi* show similar features that of *R. galegae*. According to Doc/PAGE analysis of LPSs from *A. vitis* and *A. rubi*, they had either rough-type LPS or LPS with very truncated O-chain, probably short O-chain LPS. In addition, the predominance of 3-hydroxyacids and the presence of otherwise rarely occurring 3-OH-18:1 hydroxy fatty acid are characteristic of both *R. galegae* LPS (this work) and agrobacterial LPS [29]. Thus, similar features observed in lipopolysaccharides support the theory based on 16 rRNA analysis that *R. galegae* and agrobacteria are closely related organisms.

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