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EXPERIMENTAL ARTICLES

A Comparison of the Lipopolysaccharides and O-Specific Polysaccharides of *Azospirillum brasilense* Sp245 and Its Omegon-Km Mutants KM018 and KM252

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Abstract—The lipopolysaccharides (LPSs) extracted from the outer membrane of *Azospirillum brasilense* Sp245 and its Omegon-Km mutants KM018 and KM252 with a hot aqueous solution of phenol were found to differ in the content of carbohydrates, glucosamine, and total phosphorus and in the proportion of octadecenoic and hexadecanoic acids in the lipid moieties of the LPSs. The carbohydrate moieties of the LPSs were heterogeneous in charge. The analysis of the O-specific polysaccharides (O-PSs) of the mutants KM018 and KM252 by gas–liquid chromatography, IR spectroscopy, and NMR spectroscopy showed that they are composed of the same linear pentasugar repeating units ⟶ 2)-β-D-Rha*p*-(1 ⟶ 3)-α-D-Rha*p*-(1 ⟶ 3)-α-D-Rha*p*-(1 ⟶ 2)-α-D-Rha*p*-(1 ⟶ 2)-α-D-Rha*p*-(1 ⟶ as the O-PSs of the parent strain Sp245. The reported differences in the biological activity of the LPSs of the parent and mutant strains can be due to their different chemical composition.

Key words: lipopolysaccharides, structure of O-specific polysaccharides, *Azospirillum brasilense.*

Bacteria of the genus *Azospirillum* are widespread in soils and can form associations with the roots of different plants, including grasses and cereals. Considerable research interest in azospirilla is due to their allocation to the group of diazotrophic rhizobacteria, which are able to promote plant growth.

One of the best studied species of this genus, *Azospirillum brasilense*, is predominantly associated with wheat roots [1]. Due to its high capability for endosymbiosis [2], the strain *A. brasilense* Sp245 holds great promise for practical applications and relevant fundamental studies. Among the macromolecules of the bacterial cell surface involved in the formation of associations of plants and microorganisms (including azospirilla), of great interest are polysaccharide-containing biopolymers [3, 4].

Our earlier studies [4, 5] showed that the membrane or capsular lipopolysaccharides (LPSs) of azospirilla play an important part in the colonization of host plant roots [4, 5]. The LPS-deficient kanamycin-resistant mutants KM018 and KM252, with Omegon-Km insertions in their 120-MDa plasmids [6], offer new possibilities for studying the role of LPSs in the interaction of rhizobacteria with plant roots. LPS-containing extracts of the mutant KM018 and KM252 cells exhibited the presence of only one (either external or internal) precipitin line in their double immunodiffusion tests, whereas the corresponding extract of the parent strain showed the presence of both precipitin lines. This suggested that each mutant had defects in the synthesis of only one of the two O-specific polysaccharides (O-PSs) found in the parent strain. Further studies showed that the ability of *A. brasilense* KM252 cells to adsorb on wheat seedling roots and the ability of the LPSs of the mutants KM018 and KM252 to influence the morphology of root hairs were lower than in the case of the parent *A. brasilense* Sp245 cells [5]. These data suggested that the insertion of Omegon-Km into the 120-MDa plasmid caused defects in the outer-membrane LPSs of *A. brasilense* Sp245 [6]. To understand the mechanism of action and the role of particular components of LPSs in the biological activity of bacterial membranes, it is necessary to combine relevant immunochemical studies with the isolation and analysis of individual bacterial surface antigens.

This work was aimed at the isolation and comparative analysis of the LPSs and O-PSs of the parent strain *A. brasilense* Sp245 and its Omegon-Km mutants KM018 and KM252.

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MATERIALS AND METHODS

Bacterial strains. The parent strain *Azospirillum brasilense* Sp245, isolated from surface-sterilized wheat roots [2], was kindly provided by J. Döbereiner from Empresa Brasileira de Pesquisa Agropecuaria in Rio de Janeiro, Brazil. The kanamycin-resistant mutants KM252 and KM018 of this strain with the Omegon-Km insertions in their 120-MDa plasmids [6] were provided by E.I. Katzy from the Laboratory of Microbial Genetics at the Institute of Biochemistry and Physiology of Plants and Microorganisms, Saratov.

Media and cultivation conditions were described earlier [3]. To grow the mutants, the malate-containing liquid nutrient medium was supplemented with 100 μg/ml kanamycin sulfate as the selective agent.

Isolation of LPSs and O-PSs. The respective isolation procedures are described in detail elsewhere [5]. Cells were harvested by centrifugation at 3000 *g* for 40 min. To remove capsular material, the cells were incubated in 0.15 M NaCl with 0.2% NaN_3 for 6 days. The removal of the capsular material was controlled by the double immunodiffusion technique with antibodies that were raised against whole *A. brasilense* Sp245 cells treated with glutaraldehyde. LPSs were extracted from the decapsulated cells dehydrated with acetone as described earlier [5] and then degraded with 1% acetic acid (pH 2.8) at 100°C for 4 h. O-PSs were obtained from the carbohydrate fraction of the LPSs by gel filtration on a (50 × 2.2 cm) column packed with Sephadex G-50 (the void volume V_0 = 40 ml). The eluent was 50 mM pyridine–acetate buffer (pH 4.1).

The O-PSs were separated by ion-exchange chromatography on a (20 × 1 cm) column packed with DEAE-Trisacryl M and on a (20 × 2.3 cm, V_0 = 40 ml) column packed with DEAE-Toyopearl 650M (Toyo Soda, Japan). The former column was developed with a stepwise gradient (0.005, 0.01, 0.1, 0.25, and 0.5 M) of sodium phosphate buffer (pH 6.3). The latter column was washed with 10 mM Tris–HCl buffer (pH 7.2) and then developed with a linear gradient of NaCl (10 mM to 1 M) in the same buffer. The flow rate was 0.8 ml/min. The eluted polysaccharides were desalted on a column with TSK Gel HW-40, concentrated in a vacuum rotary evaporator, and lyophilized.

During gel filtration, the eluted substances were detected with an LKB 2142 (Sweden) differential flow refractometer. During ion-exchange chromatography, elution profiles were constructed by measuring the protein concentration at 280 nm and the concentration of carbohydrates by their reaction with phenol and sulfuric acid (the reaction products have an absorption maximum at 490 nm). Spectral measurements were carried out with an SF-46 spectrophotometer (LOMO, Russia).

Carbohydrates, proteins, nucleic acids, phosphorus, and 2-keto-3-deoxyoctonic acid were analyzed by conventional methods [3].

Methyl esters of fatty acids (MEFAs) were analyzed with a Biokhrom-1 gas chromatograph (Russia) equipped with a (0.2 mm × 25 m) column with SE-54 and a flame ionization detector. The column temperature was raised from 150 to 250°C at a rate of 2.5°C/min. Fatty acids were methylated by incubating the LPSs with 2 N HCl in the presence of methanol at 84°C for 16 h [7]. The methyl esters were identified by using authentic samples purchased from Sigma (United States).

Neutral monosugars were analyzed in the form of acetylated glycosides [8]. Analysis was carried out with a Hewlett-Packard 5890 chromatograph (United States) equipped with an Ultra 2 capillary column. The column temperature was held at 180°C for 1 min and then raised to 290°C at a rate of 10°C/min. The relative content of sugars was expressed as the ratio of the peak areas.

The absolute configuration of neutral sugars was determined by the gas chromatography of their acetylated glycosides with (R)-2-octanol [9].

Aminosugars were analyzed with an AAA-339 amino acid analyzer (Czech Republic).

The IR spectra of PSs pressed with KBr were recorded with an IR-75 spectrophotometer (Carl Zeiss, Jena) with a microsample attachment.

Lipopolysaccharides were analyzed by SDS-PAAG electrophoresis [10] at 40 mA for 3 h. The concentrations of stacking and separating gels were 5 and 12.5%, respectively. Before application, the preparations to be analyzed were mixed with an aliquot of an SDS-containing sample buffer and boiled for 5 min. LPS bands were visualized by staining the developed gel slabs with silver nitrate [11].

Samples for NMR studies were lyophilized twice from 99.9% D_2O and dissolved finally in 99.96% D_2O. ^1H and ^{13}C NMR spectra were recorded at 27°C with a Bruker DRX-500 spectrometer (Germany). Chemical shifts were determined with acetone as the internal standard (δ_H = 2.225 ppm; δ_C = 31.45 ppm). The spectra were recorded and processed with the aid of the Bruker software and XWINNMR 2.1. In TOCSY and NOESY experiments, the time of mixing was 150 and 200 ms, respectively.

The results obtained in this study were subjected to statistical analysis. Confidence intervals in the paper are shown for a 95% confidence level.

RESULTS AND DISCUSSION

It is known that cultivation conditions may greatly influence the composition of bacterial LPSs [12]. Preliminary experiments showed that the parent and mutant strains of *A. brasilense* did not differ in growth rate. For this reason, LPSs were obtained from the parent *A. brasilense* Sp245 and the mutant KM018 and KM252 cells grown to the end of the exponential phase (18 h of cultivation). The mutants were grown in the presence of kanamycin. The strain purity was tested immunochemically [6].

The removal of capsule material was a necessary step of the experiments, since the monosugar compositions of the LPSs and capsular glycopolymers are often similar and could interfere with each other and thus lead to incorrect interpretation of the results [13]. The absence of capsular material on the cell surface was tested by subjecting the concentrate of the washings to the double immunodiffusion test with homologous antibodies against the whole cells of azospirilla treated with glutaraldehyde (Fig. 1). The gradual vanishing of precipitin lines in the successive washings indicated that polysaccharide-containing capsular substances were completely removed from the cell surface.

LPSs were extracted from the decapsulated and dried cells with hot aqueous phenol. The extracted LPSs concentrated in the aqueous phase, whereas the phenol phase virtually did not contain LPSs. The gel filtration of the aqueous phase on Sepharose CL-4B made it possible to separate high-molecular-weight LPSs from their degradation products, which were formed during the extraction procedure. The elution parameters of the LPSs of the parent and mutant strains (LPS_{Sp245}, LPS_{KM252}, and LPS_{KM018}) were almost identical. The LPS yield varied from 1.8 to 2.5% of the mass of dry cells.

The chemical analysis of the LPSs showed the presence of proteins and nucleic acids in only trace amounts (table), which was an indication of a high purity of the LPSs. The typical LPS component 2-keto-3-deoxyoctonic acid (KDO) was present in all the LPS preparations, although its content was low (less than 1%). A low content of KDO was also reported for other strains of *A. brasilense* and for *Azospirillum lipoferum* and was proposed to be related to a poor separation of lipid A, which is tightly bound to the polysaccharide component of LPSs [14]. This is in agreement with our data on the acid degradation of LPSs (extending the time of LPS hydrolysis did not result in precipitation of lipid A). The LPS of strain KM252 differed from those of strains Sp245 and KM018 in a 15 to 17% higher content of carbohydrates. TLC analysis showed the presence of rhamnose (Rha), glucose (Glc), and glucosamine (GlcN) in all the LPSs. The content of GlcN in the LPSs of strains Sp245 and KM252 was an order higher than in the LPS of strain KM018 (table). A high content of

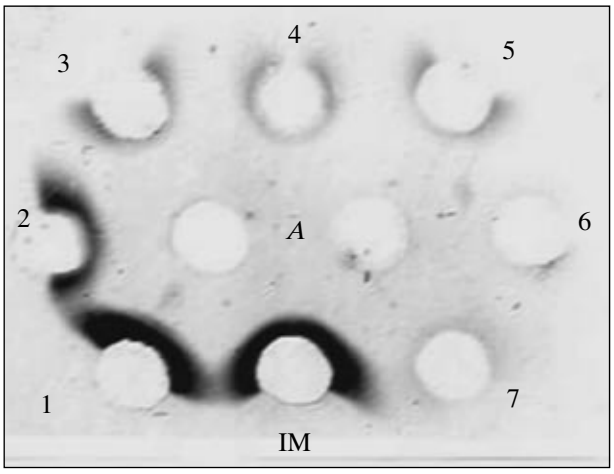

Fig. 1. The double immunodiffusion analysis of the incubation medium that was used for washing out the bacterial capsule. *A*, antibodies against the whole *A. brasilense* Sp245 cells treated with glutaraldehyde; IM, incubation medium. Numerals indicate the duration of the incubation period in days.

The chemical composition of the lipopolysaccharides of the *A. brasilense* strains Sp245, KM252, and KM018 (LPS_{Sp245}, LPS_{KM252}, and LPS_{KM018}, respectively)

Components			LPSs		
			LPS_{Sp245}	LPS^{*}_{KM252}	LPS_{KM018}
Content, wt %		Carbohydrates	65.8 ± 2.1	82.8 ± 2.50	67.9 ± 1.8
		Proteins	0.7 ± 0.06	1.3 ± 0.1	0.9 ± 0.05
		Nucleic acids	0.004	0.002	0.003
		KDO	0.6 ± 0.02	0.4 ± 0.02	0.8 ± 0.05
		Total phosphorus	2.7 ± 0.1	0.3 ± 0.02	0.5 ± 0.02
		Glucosamine	3.3 ± 0.1	4.1 ± 0.05	0.4 ± 0.2
	Percent of total MEFAs	3-Hydroxytetradecanoic acid (3OH-14:0)	42.0 ± 0.6	14.0 ± 0.7	45.0 ± 1.5
		3-Hydroxyhexadecanoic acid (3OH-16:0)	22.0 ± 0.9	17.8 ± 1.1	28.3 ± 1.1
		Hexadecanoic acid (16:0)	29.2 ± 1.3	12.8 ± 0.3	5.0 ± 0.3
		Octadecenoic acid (18:1)	6.8 ± 0.2	33.0 ± 1.3	22.7 ± 0.9
		Nanodecanoic acid (19:0)	–	12.4 ± 0.4	–

Note: Confidence intervals are shown for a 95% confidence level.

* LPS_{KM252} also contained 14:0, 16:1, and 18:0 fatty acids in amounts not exceeding 2%.

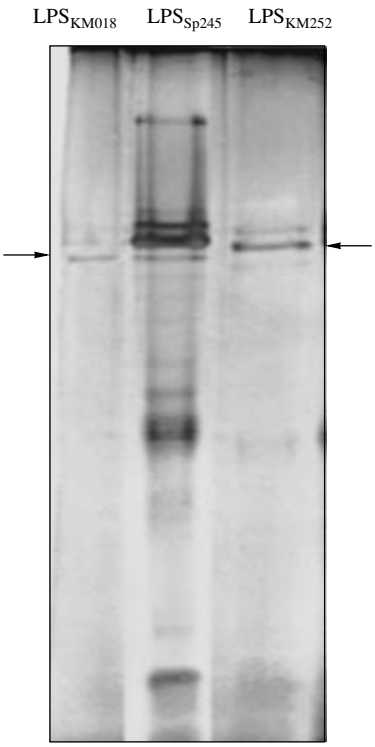

Fig. 2. Heterogeneity of the LPSs of the parent and mutant *A. brasilense* strains as is evident from electrophoretic analysis in SDS–PAAG. The arrows show the position of the major bands corresponding to polysaccharides with different negative charge densities.

Rha and Glc in the LPSs of *A. lipoferum* and *A. brasilense* was reported by Choma *et al.* [14].

The analysis of the lipid fraction of the LPSs showed the presence of saturated, unsaturated, and hydroxy fatty acids from C_{14} to C_{18} in length. The major fatty acids of LPS_{Sp245} and LPS_{KM018} were 3-hydroxytetradecanoic (3OH-14:0) and 3-hydroxyhexadecanoic (3OH-16:0) acids (approximately 70% of the total fatty acids) (table). The LPS of strain KM252 was dominated by octadecenoic (18:1) and 3-hydroxyhexadecanoic acids. The content of unsaturated octadecenoic acid in the LPSs of the mutant strains was considerably higher than in the LPS of the parent strain (where it was about 7%). The strains also differed in the content of hexadecanoic acid (16:0). Thus, the fatty acid profiles of the LPSs of the mutant strains differed from that of the parent strain. Consequently, the conformation and, hence, the biological activity of the LPSs of the mutants may differ from the LPS of the parent strain.

It should be noted that the 3-hydroxy fatty acid profile is often used as a chemotaxonomic criterion of bacteria. In general, our relevant data agree well with the data of Choma *et al.* on the fatty acid composition of the LPSs of other azospirilla [14].

The LPSs under study also differed in phosphorus content. Phosphate groups, which mainly localize in lipid A and in the core interior of LPSs, are very active cation-binding sites. The degree of ionization of negatively charged groups determines the strength of repulsion between them, the degree of hydration of the polar parts of LPS molecules, the formation of hydrogen bonds, and in the final analysis the submolecular organization of LPSs in bacterial membranes [15].

The IR spectra of the LPSs were similar. All the spectra showed the presence of intense bands at 3400 cm^{-1}, which are characteristic of the valence vibrations of associated hydroxyl groups. Absorption bands at 2900 and 799 ± 13 cm^{-1} were likely to be associated with, respectively, the valence and deformation vibrations of C–H groups. The ester group of the LPSs of strains Sp245 and KM018 gave a small peak at 1732 cm^{-1}. Absorption bands at 1640 and 1560 cm^{-1} indicated the presence of *N*-acyl hexosamine groups in the LPSs, which suggests that their lipid A components contain *N*-acylated fatty acids.

The SDS–PAAG electrophoresis of the LPSs (Fig. 2) showed that they represent mixtures of molecular S forms differing in the number of repeating units in their O-PSs. Intense bands in the upper part of the gel slab indicated a predominance of high-molecular-weight forms of the LPSs. The LPSs of the mutant strains gave a smaller number of bands (and their intensity was lower than in the case of the parent strain) than did the LPS of the parent strain. However, the position of these bands did not differ from the position of the respective bands of LPS_{Sp245}, which were produced by molecular forms differing in charge and molecular mass.

Mild acid hydrolysis, which favors cleavage of the acid-labile bond between the lipid A and the polysaccharide components of the LPS molecule, made it possible to isolate the O-PS component of the LPSs. The carbohydrate fractions of the LPSs were purified by gel filtration on Sepharose CL-4B. The amount of uncleaved LPSs (they migrated from the chromatographic column in the void volume) in these fractions was low, indicating that the conditions of acid hydrolysis were optimal for the cleavage of the LPS molecules. The fraction of O-PSs obtained by gel filtration on Sepharose CL-4B was further purified by gel filtration on Sephadex G-50. The O-PSs were eluted as one symmetric peak (Fig. 3). Elution peaks that would correspond to low-molecular-weight core polysaccharides were absent, indicating a high degree of core substitution by polysaccharide chains. All the strains had similar polysaccharide elution profiles.

Thus, the original LPSs under study were dominated by S forms. The elution of the O-PSs in the void volume indicated that their molecular mass is greater than 20 kDa. The O-PS yield varied from 32 (strains Sp245 and KM252) to 38% (strain KM018).

Ion-exchange chromatography on DEAE-Toyopearl 650M showed that the polysaccharide components of LPS_{Sp245} were heterogeneous in charge. Stepwise elution allowed the O-PSs of strain Sp245 to be separated into the fractions of acidic and neutral polysaccharides

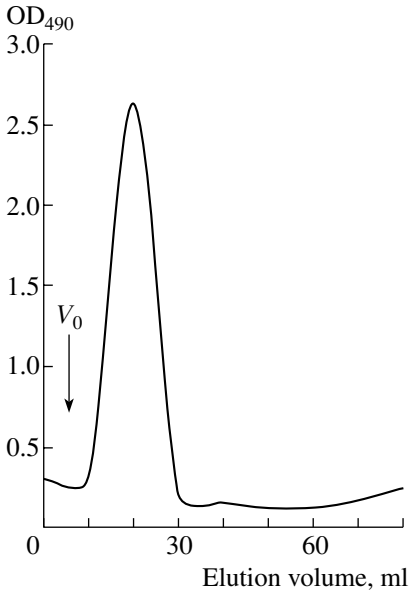

Fig. 3. The gel filtration of the polysaccharide fraction of LPS_{Sp245} on Sephadex G-50. The ordinate shows the optical density (OD_{490}) of the reaction products of the eluate with phenol and sulfuric acid.

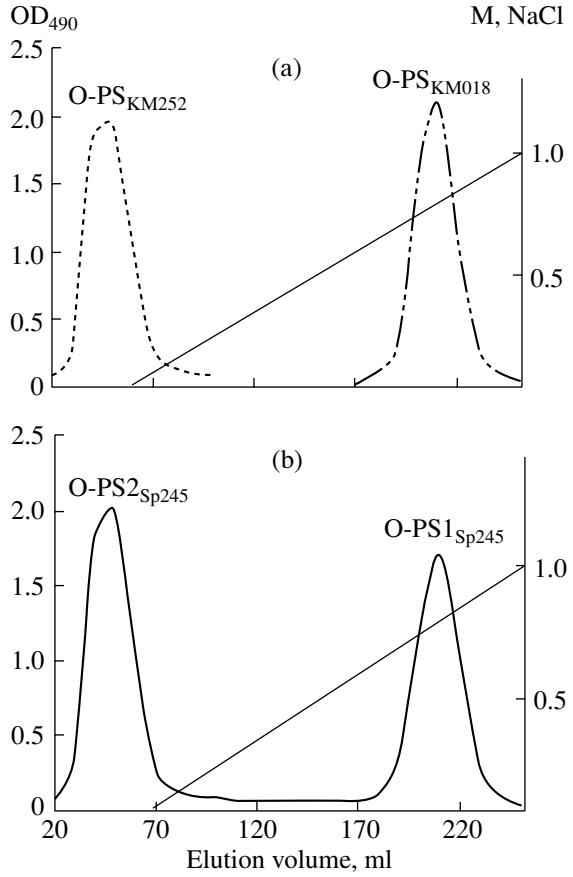

Fig. 4. The ion-exchange chromatography on DEAE-Toyopearl 650M of the O-specific polysaccharides of LPSs from (a) the mutant strains KM252 and KM018 and (b) the parent strain Sp245. $O\text{-}PS_{KM018}$ and $O\text{-}PS_{KM252}$ (panel a) are the acidic and neutral O-PSs of the mutant strains KM018 and KM252, respectively. $O\text{-}PS1_{Sp245}$ and $O\text{-}PS2_{Sp245}$ (panel b) are the acidic and neutral O-PSs of the parent strain Sp245.

(Fig. 4b). The acidic polysaccharides of strain Sp245 were further fractionated by ion-exchange chromatography on DEAE-Trisacryl into particular polysaccharides differing in negative charge density. At the same time, the LPSs of the mutant strains KM018 and KM252 contained only acidic and neutral O-PSs, respectively (Fig. 4a). Like the acidic O-PS of the parent strain Sp245, the acidic O-PS of the mutant strain KM018 was heterogeneous. The IR spectra, the monosugar compositions, and the NMR spectra of the particular fractions of $O\text{-}PS_{Sp245}$ were identical, which indicated that these fractions were also identical. Taking into account the fact that the chemical structure of the repeating unit of $O\text{-}PS_{Sp245}$ (the D-rhamnose–based pentasugar) was determined earlier [16], we concentrated on the study of the O-PSs of the mutant strains.

The acid hydrolysis of the O-PSs of the mutant strains gave rise to the deoxysugar rhamnose alone, which was identified by paper and gas–liquid chromatographies. Like the rhamnose of the O-PS of strain Sp245 [18], the rhamnose of the mutant strains KM018 and KM252 had a D configuration. It should be noted that deoxyhexoses (for instance, the rhamnose of the O-PS of *A. lipoferum* SpBr17) usually have an L configuration [17].

The IR spectra of the O-PSs of the mutant strains were almost identical and had bands of middle intensity at 834 cm^{-1}, which indicated that the monosugar residues of the O-PSs are mainly bound by an α-glycosidic bond. The presence of low-intensity spectral bands at 885–890 cm^{-1} suggested that some monosugar residues were also bound by a β-glycosidic bond. The broad band at 2933 cm^{-1} was likely to be due to the valence vibrations of methyl groups in rhamnose residues [19].

The complete structure of the O-PSs was determined by NMR spectroscopy. The ^{13}C NMR spectra of the O-PSs of the mutant strains were identical and had signals corresponding to the methyl groups of five Rha residues at 17.8–17.9 ppm, the signals of five anomeric carbon atoms at 97.8–103.3 ppm, and 20 signals of the secondary carbon atoms of monosugar cycles in the region 68.4–79.0 ppm. The ^{1}H NMR spectra of the O-PSs of the mutant strains were also identical (Fig. 5) and had signals corresponding to the methyl groups of five Rha residues at 1.3–1.33 ppm, the signals of five anomeric protons at 4.82–5.20 ppm, and signals of other protons in the region 3.43–4.25 ppm. In general, the NMR spectra of the O-PSs of the mutant strains were identical to those of the O-PS of the parent strain. Consequently, the O-PSs of the mutants had the same pentasugar repeating unit as the O-PS of the parent strain, namely,

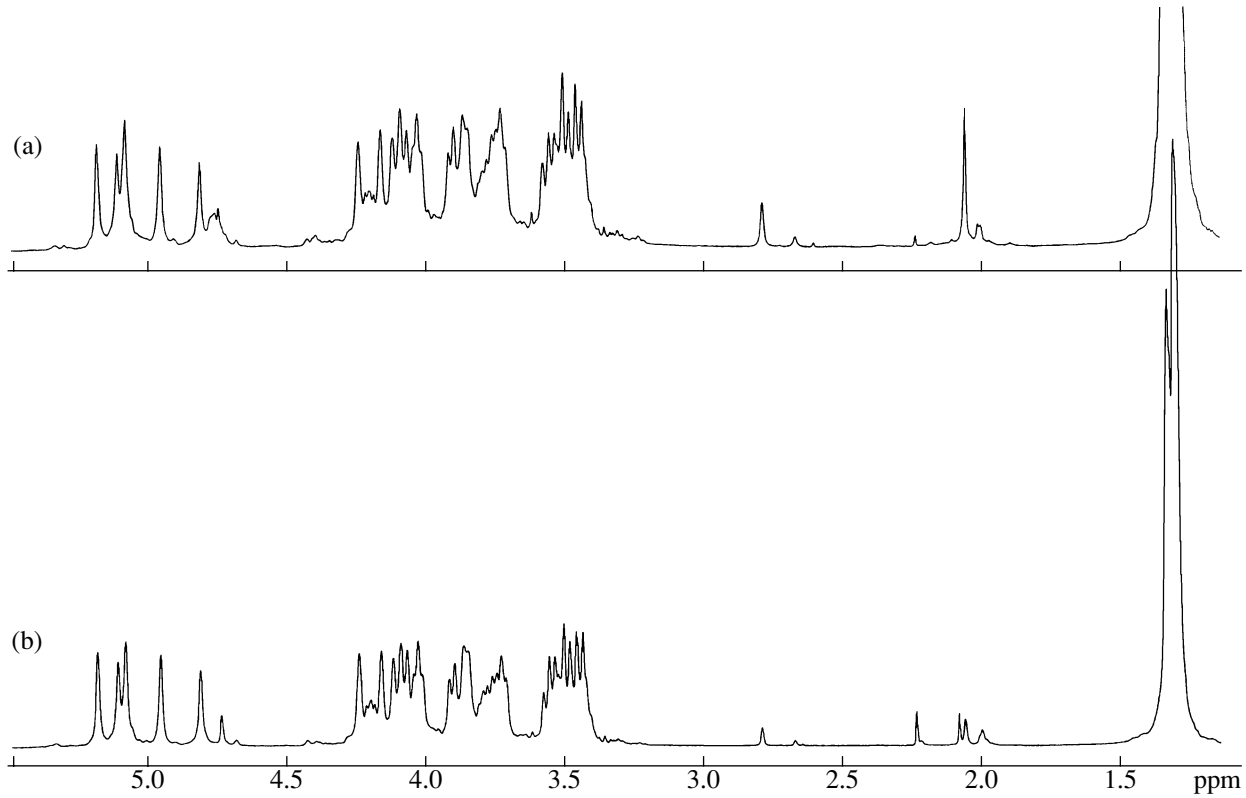

Fig. 5. The ^{1}H NMR spectra of (a) O-PS$_{KM252}$ and (b) O-PS$_{KM018}$ derived from the LPSs of the mutant strains KM252 and KM018, respectively.

⟶ 2)-β-D-Rha*p*-(1 ⟶ 3)-α-D-Rha*p*-(1 ⟶ 3)-α-D-Rha*p*-(1 ⟶ 2)-α-D-Rha*p*-(1 ⟶ 2)-α-D-Rha*p*-(1 ⟶

The charge heterogeneity of the polysaccharide components of the LPSs may be related to the presence of phosphate groups as minor substituents. This suggestion is in agreement with the different phosphorus contents of the LPSs. The electrophoretic heterogeneity of the O-PSs suggests that they may also differ in the degree of polymerization, as was observed by Bakholdina *et al.* [12] for the O-PSs of *Yersinia pseudotuberculosis.*

To conclude, the repeating units of the O-PSs of the Omegon-Km mutants of *A. brasilense* Sp245 have the same structure as the repeating unit of the O-PS of the parent strain. Small differences in the chemical structure of the hydrophobic moieties of the LPSs may be responsible for differences in their biological activity observed in the root hair deformation test with wheat seedlings [5].

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