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EXPERIMENTAL ARTICLES

Determination of the Structure of the Repeated Unit of the *Azospirillum brasilense* SR75 O-Specific Polysaccharide and Homology of the *lps* Loci in the Plasmids of *Azospirillum brasilense* strains SR75 and Sp245

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Abstract—The structural identity of the repeated unit in O-specific polysaccharides (OPSs) present in the outer membrane of strain SR75 of the bacterium *Azospirillum brasilense*, isolated from wheat rhizosphere in Saratov oblast, and the previously studied OPSs of *A. brasilense* strain Sp245, isolated from surface-sterilized wheat roots in Brazil, has been demonstrated. Plasmid profiles, DNA restriction, and hybridization assays suggested that *A. brasilense* strains SR75 and Sp245 have different genomic structures. It was shown that homologous *lps* loci of both strains were localized in their plasmid DNA. This fact allows us to state that, despite their different origin, the development of the strains studied was convergent. Presumably, the habitation of these bacteria in similar ecological niches influenced this process in many respects.

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

Gram-negative bacteria from the genus *Azospirillum* are intensively studied associated partners of a wide range of plant species growing in diverse climatic zones [1]. Without doubt, the inclusion of *Azospirillum* in a group of microorganisms that stimulate plant growth and yield is justified. However, the mechanism involved in the formation of efficient plant–*Azospirillum* associations remains to be clarified. The important role of surface bacterial structures and glycopolymers, such as exopolysaccharides, flagella, and capsular polysaccharides, in adsorption to plant roots and their colonization has been demonstrated [2, 3]. However, reports on the functions of lipopolysaccharides (LPSs), major components of the outer membrane of gram-negative bacteria, are only beginning to emerge. Indirect evidence of LPS involvement in the adsorption of *Azospirillum* cells on wheat roots has been obtained, and the ability of LPSs to induce changes in root hair morphology discovered [4]. The use of genetic methods in studies of the mechanism underlying the formation of plant–microbial associations is very promising. It has been demonstrated that three plasmids of model *A. brasilense* strains Sp245 and Sp7 are involved in synthesizing substances important for the formation and function of rhizocenoses [5]. In particular, four loci determining the synthesis of lipopolysaccharides (LPSs) were found in 120-MDa plasmid (p120) Sp245; two of these loci are also necessary for the synthesis of Calcofluor-binding polysaccharides (CBPSs, Cal^+ phenotype) [6]. Omegon mutants of *A. brasilense* Sp245, defective in the synthesis of CBPSs and of the LPSs characteristic of the wild type, were also constructed, namely, $LpsI^-$ Cal^-, $LpsI^-$, $LpsII^-$ Cal^-, and $LpsII^-$ [6]. Charge heterogeneity has been detected in the carbohydrate moiety of the LPS of *A. brasilense* strain Sp245. However, NMR spectroscopy of the O-specific polysaccharides (OPSs) of these *Azospirillum* strains detected no differences in the structure of their repeated units [7]. Thus, determination of the fine chemical structure of *Azospirillum* OPSs is a necessary and important stage in studies of plant–microbial interactions at the molecular level.

The incentive for this study was provided by the discovery of the serological activity of a preparation of *A. brasilense* SR75 LPS, as was demonstrated by the cross-reaction of double immunodiffusion in agarose (two precipitation bands) with rabbit antibodies to whole *A. brasilense* Sp245 cells treated with glutaraldehyde (Fig. 1). This was the first time that such a high level of serological relatedness of azospirilla had been found and is particularly interesting in view of the fact that strains SR75 and Sp245 were recovered from dif-

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ferent climatic zones, even different continents. *A. brasilense* Sp245, one of the most thoroughly studied *Azospirillum* strains, was first described in Brazil by Döbereiner *et al.* This strain is known as a wheat endosymbiont, displaying a high potential for colonizing plant roots [8], producing phytohormones, and inducing branching of roots and root hairs [9]. The bacteria *A. brasilense* SR75, first isolated from the wheat rhizosphere in Saratov oblast (Russia) [10], are actively adsorbed into wheat roots [3] and have surface heteropolysaccharides capable of interacting specifically with wheat germ agglutinin and inducing deformations of wheat root hairs [11].

The goal of this work was to compare OPS structures and genetic loci determining LPS formation of two *A. brasilense* strains isolated from wheat in regions with moderate and tropical climates.

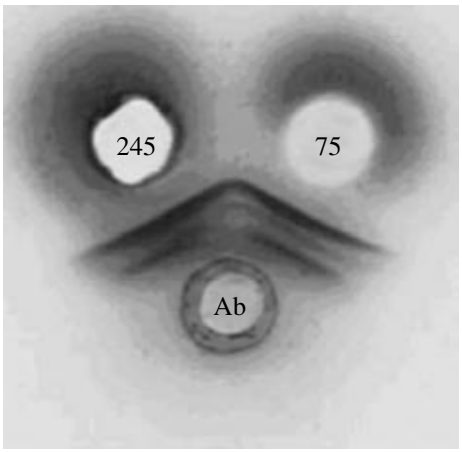

Fig. 1. Double radial immunodiffusion of the lipopolysaccharides of *A. brasilense* Sp245 (245) and *A. brasilense* SR75 (75) with rabbit antibodies (Ab) to whole *A. brasilense* Sp245 cells treated with glutaraldehyde.

MATERIALS AND METHODS

The bacterial strains and plasmids used in this study are listed in the table.

Conditions for growing bacteria. The cultures of *A. brasilense* to be used for LPS isolation were grown in a liquid malate–salt medium containing vitamins [12] at 30°C. *Escherichia coli* was cultivated in LB medium [13] at 37°C. When necessary, the medium was supplemented with kanamycin (Km) at a concentration of 25 μg/ml.

LPS and OPS isolation. Capsular material was washed off the surface of the *A. brasilense* SR75 cells in a 0.15 M NaCl solution supplemented with NaN_3. The completeness of capsule removal was controlled by a double immunodiffusion test with antibodies to whole *Azospirillum* cells treated with glutaraldehyde. LPSs were isolated from acetone-dried capsule-free cells (the protocol was described in [4]) and hydrolyzed with 1% acetic acid (pH 2.8) for 4 h at 100°C. The hydrolysate was centrifuged at 12000 *g* for 20 min, to separate the sediment of lipid A. The OPSs were separated from the water-soluble fraction by gel chromatography in a column (50 × 2.2 cm; V_0 = 40 ml) with Sephadex G-50 (Pharmacia, Sweden) using a 0.05 M pyridine–acetate buffer (pH 4.1) as an eluant. The materials were detected using an LKB 2142 (Sweden) differential flow refractometer.

Bacterial strains and plasmids used

Strain or plasmid	Characteristic	Source
Strains:		
Azospirillum brasilense		
Sp245	Wild type, isolated in Brazil from wheat roots	[8]
SR75	Wild type, isolated in Russia from wheat seedlings	[10]
Escherichia coli		
DH1	*supE44*, *hsdR17*, *recA1*, *endA1*, *gyrA96*, *thi-1*, *relA1*, recipient of cloned *A. brasilense* genes	[13]
Plasmids:		
pOmegon-Km-*lps*348X	*Xho*I fragment of p120 carrying Omegon-Km (3.8 kbp) and *lps* and *cal* loci from *A. brasilense* KM348 (LpsI⁻ mutant of Sp245), 18.8 kbp, Km^R. The *Bam*HI fragment (15 kbp) of this plasmid was used as the hybridization probe	[6]
pOmegon-Km-*fla*048X	*Xho*I fragment of p120 carrying Omegon-Km and fla/swa loci from *A. brasilense* SK048 (Fla⁻ Swa⁻ mutant of Sp245), 12.1 kbp, Km^R. The *Bam*HI fragment (8.3. kbp) of this plasmid was used as the hybridization probe	[21]

Analytical methods. The monosaccharide composition was assayed by gas–liquid chromatography (GLC) of polyol acetates following complete OPS hydrolysis with 2 M CF_3COOH (121°C, 2 h), reduction with $NaBH_4$, and acetylation, as well as by GLC–mass spectrometry of partially methylated polyol acetates, as described in [14]. The samples were analyzed in a Hewlett Packard 5890 chromatograph (equipped with a capillary column containing Ultra 2 as stationary phase) in a temperature gradient from 180°C (1 min) to 290°C (at a heating rate of 10°C/min) and in a Hewlett Packard 5989 chromatograph–mass spectrometer using an HP-1 (Hewlett Packard, USA) capillary column.

The absolute configuration of neutral sugars (in the form of acetylated glycosides with the optically active alcohol (*R*)-2-octanol) was determined by GLC [15]. The conditions were the same as indicated above.

The content of carbohydrates, 2-keto-3-deoxyoctonic acid (KDO), proteins, nucleic acids, and phosphorus were determined colorimetrically according to the conventional protocols described in [12].

In order to perform an NMR assay, the samples were freeze-dried twice from 99.9% D_2O and dissolved in 99.9% D_2O. ^1H- and ^{13}C-NMR spectra were recorded in a Bruker DRX-500 (Germany) spectrometer at 27°C. The chemical shifts were determined using acetone as an internal standard (δ_H 2.225, δ_C 31.45). The spectra were recorded using standard Bruker software; the data were read and processed using the program XWINNMR 2.1. The mixing time in the TOCSY and NOESY experiments amounted to 150 and 200 ms, respectively.

The results of all the experiments were processed statistically, and the confidence intervals were 95%.

Handling of DNA. The plasmids were visualized by in situ cell lysis and gel electrophoresis, as described by Eckhardt [16]. The total and plasmid DNA was isolated and purified from agarose gel and blotted from the gel onto Hybond-N^+ (Amersham) nylon membranes in accordance with the standard procedures [13]. The DNA fragments were labeled with peroxidase and Southern hybridized using an ECL (enhanced chemiluminescence) system for gene detection in accordance with the manufacturer's (Amersham Pharmacia Biosciences) recommendations.

RESULTS AND DISCUSSION

Determining the structure of the repeated unit of the *A. brasilense* SR75 OPS. LPSs were extracted from the acetone-dried capsule-free cells of *A. brasilense* SR75 with hot 45% aqueous phenol. The yield of LPS preparation after chromatographic purification amounted to 1% of the dry cell weight. The assays demonstrated that the LPS isolated contained 53% carbohydrates, 3.3% KDO (the component of the core region, specific to LPSs of gram-negative bacteria), 0.1% phosphorus, and trace amounts of protein and nucleic acids.

Following mild acid hydrolysis of the *A. brasilense* SR75 LPS, lipid A was sedimented by centrifugation and the OPS isolated from the water-soluble fraction by gel filtration in a column with Sephadex G-50; the yield of OPS was 32% of the total LPS. The relatively high yield of the carbohydrate moiety of the LPS and the elution profile of the OPS suggested predominance of the S-form of molecules in the initial preparation. As was demonstrated in [17], a similar LPS macromolecular organization is characteristic of *A. brasilense* Sp245.

After methanolysis, GLC was used to identify saturated, hydroxy, and unsaturated fatty acids with chain lengths of C_{12} to C_{19} in the lipid A fraction of the LPS of *A. brasilense* SR75 (Fig. 2). The main fatty acids with respect to the content were 3-hydroxytetradecanoic (3-OH-$C_{14:0}$), hexadecenoic ($C_{16:1}$), hexadecanoic ($C_{16:0}$), 3-hydroxyhexadecanoic (3-OH-$C_{16:0}$), octadecenoic ($C_{18:1}$), and nanodecanoic ($C_{19:0}$) acids. Their ratio, calculated as a percentage of the total peak area on the chromatogram, amounted to 18.1 : 6.7 : 4.7 : 14.9 : 41.3 : 9.8, respectively.

GLC analysis of the components of the OPS hydrolysate in the form of polyol acetates allowed us to identify a neutral 6-deoxysugar, rhamnose (Rha). Determination of its absolute configuration showed that this monosaccharide had a *D*-form. GLC–mass spectrometry of the partially methylated polyol acetates within the OPS identified 2-substituted and 3-substituted rhamnose residues at a ratio of 3 : 2. These data suggested that the polysaccharide is a liner pentarhamnane, while its constituent monosaccharides have a pyranose form.

The complete OPS structure was determined using spectroscopy. The ^{13}C-NMR spectrum (Fig. 3) contained signals of 5 anomeric carbon atoms in the region 97.8–103.3 ppm, methyl groups of 5 Rha residues at 17.8–17.9 ppm, and 20 signals of the secondary carbon atoms of monosaccharide rings in the region of 68.4–79.0 ppm. The ^1H-NMR spectrum of the polymer studied displayed the signals of five anomeric protons in the region 4.82–5.20 ppm, five Rha methyl groups at 1.30–1.33 ppm, and of the rest protons in the region of 3.43–4.25 ppm. Thus, the NMR spectra of the *A. brasilense* SR75 OPS were identical to the corresponding spectra of *A. brasilense* Sp245 (the primary structure of its repeated unit was established in [17]). Consequently, the OPSs of these two strains consist of the same linear pentasaccharide repeats containing only *D*-Rha residues and displaying the following structure:

$$\rightarrow 2)\text{-}\beta\text{-D-Rha}p\text{-}(1 \rightarrow 3)\text{-}\alpha\text{-D-Rha}p\text{-}(1 \rightarrow 3)\text{-}\alpha\text{-D-Rha}p\text{-}(1 \rightarrow 2)\text{-}\alpha\text{-D-Rha}p\text{-}(1 \rightarrow 2)\text{-}\alpha\text{-D-Rha}p\text{-}(1 \rightarrow$$

The identical structure of the polysaccharide in bacteria belonging to different genera and the similarity to polysaccharide antigens found in mammals are frequently attributed to the phenomenon of mimicry, which allows evasion of the immune system of the macroorganism. However, there are numerous examples of bacterial strains belonging to the same species and producing identical OPSs. In the case considered here, the similarity between the OPSs correlates with the presence of a common macropartner of these strains, namely, wheat plants. Although azospirilla are not restricted to one host, they nonetheless interact with plants in a selective manner [18].

The presence of rhamnose (either D or L) in OPSs as a predominant (or sole) component has also been demonstrated for a number of soil nitrogen-fixing bacteria, as well as for certain phytopathogenic bacteria [19, 20]. This sugar is widely prevalent among microorganisms interacting with plants. Presumably, this circumstance suggests the important role of rhamnose in recognition and interactions between plants and microorganisms.

Thus, the data obtained suggest that, despite their different origins, the strains under investigation in this study developed in a convergent manner. Possibly, habitation of the bacteria in question in similar ecological niches influenced this process in many respects.

Comparative DNA analysis of *A. brasilense* strains SR75 and Sp245. The discovered identity of the OPS structures of SR75 and Sp245 set forth the problem of comparative analysis of their DNA.

The data obtained demonstrate essential differences in the genomic structures of *A. brasilense* strains SR75 and Sp245. For example, hydrolysis of the total DNA of strains SR75 and Sp245 with restriction endonucleases produces different sets of restriction fragments (Fig. 4a). The plasmid profiles of *A. brasilense* SR75 and Sp245 lack replicons with similar molecular weights (Fig. 5a). An ECL hybridization with the 8.3-kbp fragment of p120, flanking the Omegon insertion in the Sp245 mutant, which lost its polar flagellum and the ability to swarm (table), failed to detect significant homology in the SR75 DNA (Fig. 4c).

A fragment of p120 that contained at least two *lps* loci and one *cal* locus [6] was also used as a probe in DNA hybridization. In order to produce the *lps* probe, recombinant plasmid pOmegon-Km-*lps*348X [6] (table) was hydrolyzed at the Omegon's external borders with *Bam*H1 restriction endonuclease. The big *Bam*H1 fragment (with a length of 15 kbp) corresponding to the p120 DNA per se was labeled with peroxidase and used in hybridization reactions with the total DNA and native plasmids of *A. brasilense* SR75 (Figs. 4b and 5b). (Note that the p120 fragment cloned within pOmegon-Km-*lps*348X lacks any *Bam*H1 sites of its own [6].) As a result, a significant homology to the used *lps* probe was found in the total DNA of

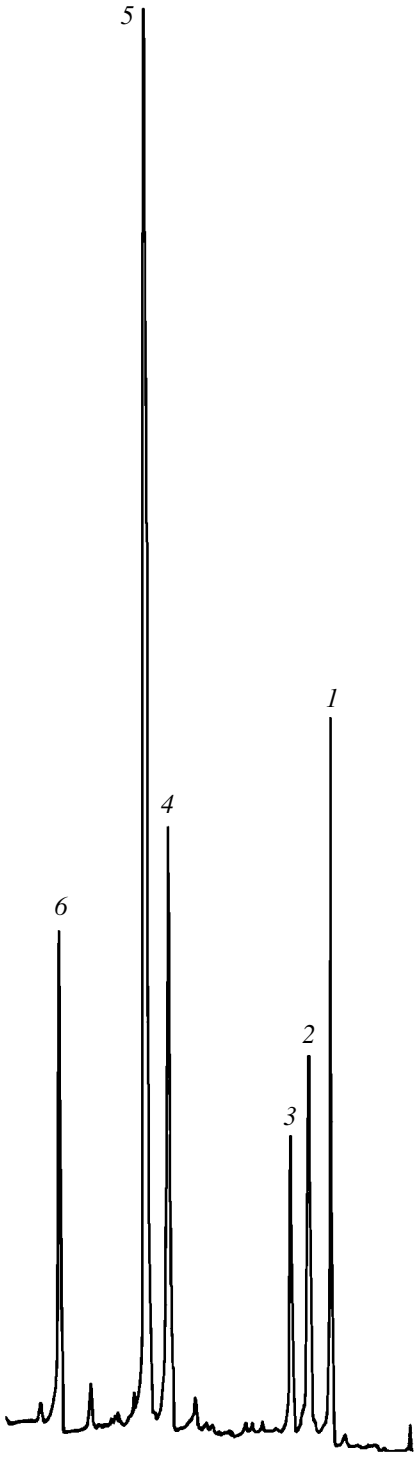

Fig. 2. GLC profile of methyl esters of the fatty acids of *A. brasilense* SR75 lipid A: (*1*) 3-OH-$C_{14:0}$; (*2*) $C_{16:1}$; (*3*) $C_{16:0}$; (*4*) 3-OH-$C_{16:0}$; (*5*) $C_{18:1}$; and (*6*) $C_{19:0}$.

A. brasilense SR75 (Fig. 4b) and the two megaplasmids of this strain (Fig. 5b).

Note that the homology to the p120 fragment used in this study has previously been detected in the

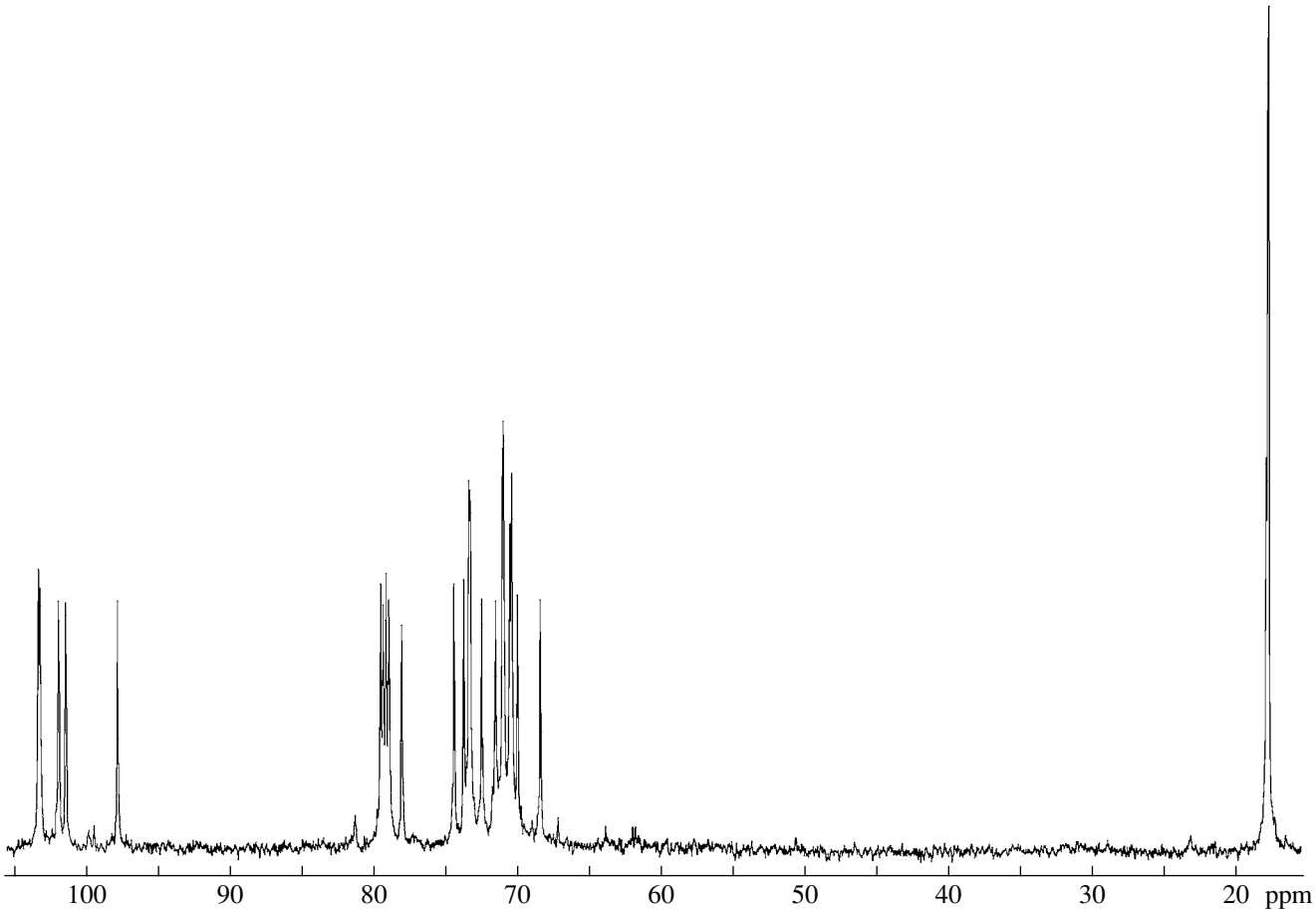

Fig. 3. ^{13}C-NMR spectrum of the O-specific polysaccharide of *A. brasilense* SR75.

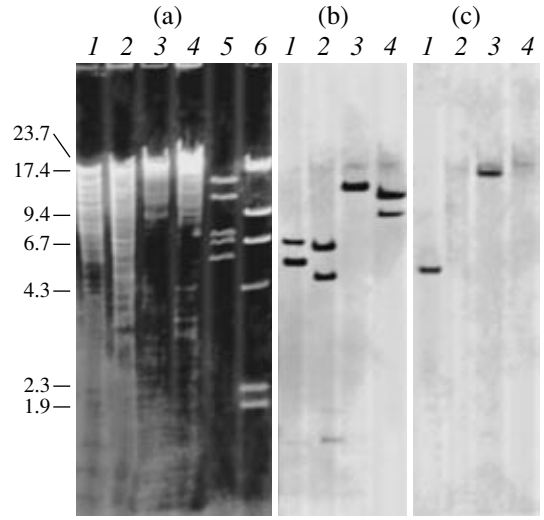

Fig. 4. Search for homology to fragments of *A. brasilense* Sp245 p120 in the total DNA of *A. brasilense* SR75. (a) (*1* and *2*) *Eco*RI and (*3* and *4*) *Bam*HI restriction fragments of the total DNA of strains (*1* and *3*) Sp245 and (*2* and *4*) SR75; (*5* and *6*) molecular markers, *Bam*HI and *Hin*dIII restriction fragments of λ phage DNA, respectively. (b) and (c) Southern hybridization of the gel from panel a with peroxidase-labeled p120 DNA of plasmids pOmegon-Km-*lps*348X and pOmegon-Km-fla048X, respectively. Positions and lengths (kbp) of some λ phage DNA restriction fragments are shown on the left.

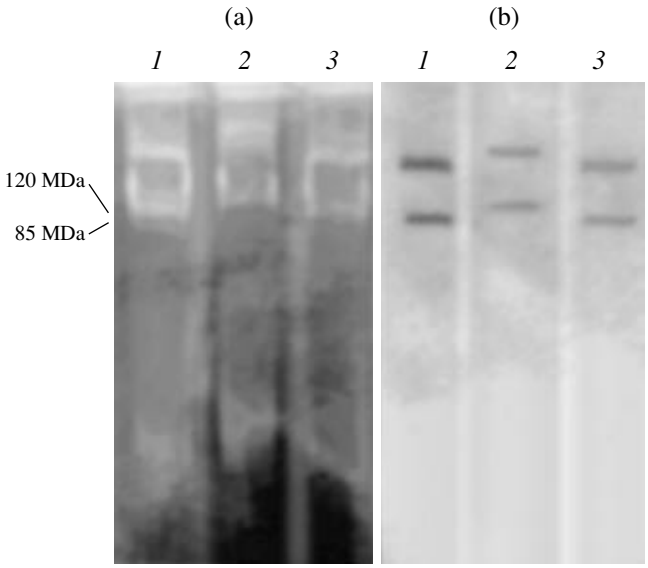

Fig. 5. Homology to the *lps* probe, a fragment of *A. brasilense* Sp245 p120 in the plasmids of *A. brasilense* SR75. (a) The plasmid profiles of (*1* and *3*) Sp245 and (*2*) SR75. (b) Southern hybridization of the gel from panel (a) with peroxidase-labeled p120 DNA of plasmid pOmegon-Km-*lps*348X. Molecular weights of plasmid DNA (MDa) are shown on the left.

90-MDa plasmid (pRhico) of the standard strain *A. brasilense* Sp7 (table) [21]. This result may be accounted for by the involvement of both p120 and pRhico in the formation of the polar flagellum and exopolysaccharides [5]. Nonetheless, the total DNA of Sp7 contains restriction fragments exhibiting only a weak positive signal when hybridized with the *fla* and *lps* loci of p120. In particular, this complies with the essential distinctions between the OPS structures of Sp245 and Sp7 (our unpublished data). In contrast, signals of virtually near-identical intensities, detected by hybridization with the *lps* and *cal* loci of p120, as well as the close total lengths of the *Eco*RI- and *Bam*HI-positive restriction fragments of the cellular DNA of Sp245 and SR75 (Fig. 4b), suggest a high degree of homology between the corresponding genetic loci of the strains in question.

Thus, this study has demonstrated the identity of OPS structures of *A. brasilense* strains Sp245 and SR75, isolated from the wheat roots or seedlings in different climatic zones, while the plasmids of these strains, having different genomic structures, contain highly homologous loci that presumably determine LPS production. The discovered fact demonstrates that it is impossible to identify the strains of these bacteria based solely on their LPSs and that a combination of chemotyping and other immunochemical and molecular biological methods is required.

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