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EXPERIMENTAL ARTICLES

Chemical Composition and Immunochemical Characteristics of the Lipopolysaccharide of Nitrogen-Fixing Rhizobacterium *Azospirillum brasilense* CD

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Abstract—The chemical composition of the lipopolysaccharide of the associative diazotrophic rhizobacterium *Azospirillum brasilense* Cd has been studied. Among the main components of the hydrophobic part of the lipopolysaccharide, we identified 3-hydroxytetradecanoic, hexadecenoic, 3-hydroxyhexadecanoic, hexadecanoic, octadecenoic, and nanodecanoic fatty acids; the carbohydrate part contained rhamnose, galactose, and mannose. Polyclonal antibodies against the preparation under study were raised in rabbits. Serological relations between *A. brasilense* Cd and other strains of *Azospirillum* spp. were studied using double radial immunodiffusion and enzyme-linked immunosorbent assay.

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Bacteria of the genus *Azospirillum* are widespread in soils and comprise diverse diazotrophic rhizobacteria stimulating plant growth and development. Polysaccharide components of the surface azospirilla play an important role in the formation of associations with plant roots and in the interactions with other rhizobacteria [1, 2]. However, the chemical nature of glycopolymers of the outer membrane and their participation in such processes remain poorly understood.

Analysis of the structural and functional characteristics of lipopolysaccharides involved in processes whereby bacteria and plant hosts recognize and interact with each other holds an important position among the major avenues of research into the associative potential of these organisms. The molecule of lipopolysaccharides (LPSs), which constitute the major component of the cell wall of gram-negative bacteria, consists of three structurally distinct parts known as lipid A, core oligosaccharide, and O-specific polysaccharide (OPS), or O-antigen. OPS is the portion of prime interest: due to its exposure on the surface of the bacterial membrane and the presence of specific antigen determinants, OPS may be used for the immunological identification of bacteria.

The immunochemical properties of the cell surface of bacteria of the genus *Azospirillum* have been studied in detail [3–6]. However, the serological classification of azospirilla based on the structure of O-antigen remains to be developed. The structures of OPSs have been determined for only four strains belonging to three species of the genus *Azospirillum*: *A. lipoferum* SpBr17, *A. brasilense* Sp245, *A. irakense* KBC1, and *A. lipoferum* Sp59b [7–10]. LPS structure is a conventional chemotaxonomic criterion, and, for this reason, clarifying it for *Azospirillum* spp. is undoubtedly of great importance.

In this work, we sought to study LPS of the bacterium *A. brasilense* Cd and compare the immunochemical properties of *Azospirillum* strains using polyclonal rabbit antibodies against LPS_{Cd}.

MATERIALS AND METHODS

Bacterial strains used in this work included *Azospirillum brasilense* Cd (ATCC 29710) [11], Sp7 (ATCC 29145) [12], Sp245 [13], and *A. lipoferum* Sp59b [12]. The bacteria were cultured in liquid malate–salt medium supplemented with vitamins [1] until the completion of the exponential phase of growth. The cultures were incubated in an ANKUM-2M fermentor (Russia) at 30°C. The cells were pelleted by centrifugation.

Capsular material was washed off the cell surface using a 0.15 M solution of NaCl (containing 0.02% NaN_3), by incubation for 5 days, with daily replacements of the solution with a fresh portion. LPS was isolated from acetone-dried capsule-free cells by aqueous phenol (the Westphal technique), as described previously [14]. The high-molecular-weight fraction of

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LPS was isolated from the extract using gel filtration on a 55 × 1.8 cm Sepharose CL-4B column (Pharmacia, Sweden) with a void volume of 40 ml. The column was eluted with 0.025 M NH_4HCO_3 (pH 8.3). LPS was degraded by incubation with 1% acetic acid (pH 2.8) at 100°C for 4 h. The OPS fraction was isolated in the course of chromatography of the carbohydrate moiety on a 50 × 2.2 cm Sephadex G-50 column (Pharmacia) with a void volume of 40 ml. The column was eluted with 0.05 M pyridine–acetate buffer (pH 4.1). The materials were detected using a differential flow refractometer (model 2142; LKB, Sweden).

Colorimetric measurements of the content of carbohydrates, 2-keto-3-deoxyoctulosonic acid (KDO), proteins, nucleic acids, and phosphorus were performed using conventional procedures described by us previously [1] on an SF-46 spectrophotometer (LOMO, Russia).

Monosaccharide composition of polysaccharides was studied by gas–liquid chromatography (GLC) of polyol acetates following complete OPS hydrolysis by 2 M CF_3COOH (120°C, 2 h), $NaBH_4$ reduction, and acetylation. GLC–mass spectrometry of partially methylated polyol acetates [15] was then performed. The samples were analyzed on a Hewlett-Packard model 5890 chromatograph (equipped with a capillary column; Ultra 2 served as a stationary phase; the temperature gradient was from 180°C (1 min) to 290°C, and the heating rate, 10°C/min) and a Hewlett-Packard model 5989 chromato-mass-spectrometer (equipped with an HP-1 capillary column) (United States). The absolute configuration of neutral sugars was determined by GLC of acetylated glycosides (formed with the optically active alcohol, (R)-2-octanol). The conditions of the chromatography were the same as described above. Methylation of fatty acids contained in lipid A of the LPS of *A. brasilense* Cd was performed according to the method described in [15]. The samples were analyzed on a Biochrom-1 (Germany) chromatograph equipped with a 25-m capillary column (OV-101 served as a stationary phase). The temperature was programmed in the range 130–250°C(heating rate, 4°C/min); the temperature of the evaporator and detector were 250 and 260°C, respectively; the flow rate of the helium carrier gas equaled 1.3 cm^3/min (split ratio, 1 : 50). The content of each acid was expressed as a percentage of the total amount of all fatty acid methyl esters detected chromatographically. Fatty acid methyl esters were identified using Supelco standards (United States).

Polyclonal antibodies ($ABLPS_{Cd}$) were raised in rabbits against the chromatographically purified LPS_{Cd} preparation. The rabbits were immunized three times at biweekly intervals by successive injection of 0.5, 1.0, and 1.5 mg LPS_{Cd} into the popliteal lymph nodes. The antigen was mixed 1 : 1 with complete Freund's adjuvant. Blood for antibody separation was taken one week after the last immunization. Immunoglobulin G fractions were obtained from the antisera by ammonium sulfate precipitation [18].

Immunodiffusion analysis was performed in a 1% agarose gel applied onto clean glass plates, according to the standard procedure. The gels were washed repeatedly and stained with Coomassie Brilliant Blue R-250 (Serva, Germany).

SDS-PAGE of LPS preparations was performed in 12.5% polyacrylamide [16]. LPS was visualized by staining the gels with silver [17]. The separated components were transferred onto 0.2-μm nitrocellulose filters (Schleicher & Schuell) by electroblotting. Immunodetection was achieved by incubating the blots with $ABLPS_{Cd}$; horseradish peroxidase conjugated with goat anti-rabbit antibodies and 3,3'-diaminobenzidine were used for visualization.

Enzyme-linked immunosorbent assay (ELISA) was performed in 96-well plates. *o*-Phenylene diamine and hydrogen peroxide were used as a substrate reagent. Measurements of optical density of the samples were performed at 490 nm using an AIF-Ts-01S plate reader (ILIP, St. Petersburg, Russia).

RESULTS AND DISCUSSION

LPS was extracted from dry biomass (acetone powder of capsule-free bacterial cells) of the collection culture *A. brasilense* Cd with a yield of 3.2% (w/w). Studies of the biopolymer composition of the preparation using component-specific reactions demonstrated that the content of carbohydrates amounted to 30% (w/w); KDO accounted for 3.5%; trace amounts of phosphorus were also detected. The preparation contained insignificant admixtures of protein and nucleic acids (of less than 0.1%).

Mild acidic degradation of LPS_{Cd}, followed by centrifugation, produced lipid A residue. Sephadex G-50 chromatography separated carbohydrate-containing materials from the supernatant into OPS fractions (yield, 37% of LPS weight) and core oligosaccharide (Fig. 1). As follows from the data of Fig. 1, OPS was eluted as a single symmetrical peak. A small peak present in the chromatogram (peak *2*) corresponded to the low-molecular-weight fraction of the core oligosaccharide. The character of the elution curve led us to conclude that the core is highly substituted with polysaccharide chains. The ratio of the isolated fractions and the appearance of the SDS-PAGE electrophoretogram of LPS_{Cd} (Fig. 2) indicated that the starting preparation was largely represented by S-forms. Such macromolecular organization was documented previously for LPSs of other *Azospirillum* strains [9, 10, 19, 20].

Methanolysis of the lipid A fraction followed by GLC made it possible to identify saturated, unsaturated, and hydroxyl-substituted C_{12}–C_{19} fatty acids (Fig. 3). 3-Hydroxytetradecanoic (3-OH-$C_{14:0}$), hexadecenoic ($C_{16:1}$), hexadecanoic ($C_{16:0}$), 3-hydroxyhexa-

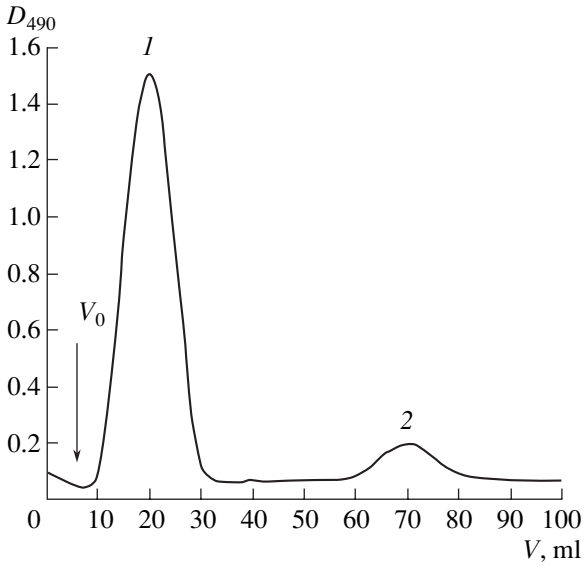

Fig. 1. Elution profile of the carbohydrate portion of the O-antigen of *A. brasilense* Cd (Sephadex G-50 gel filtration): *1*, O-specific polysaccharide fraction; *2*, core oligosaccharide fraction.

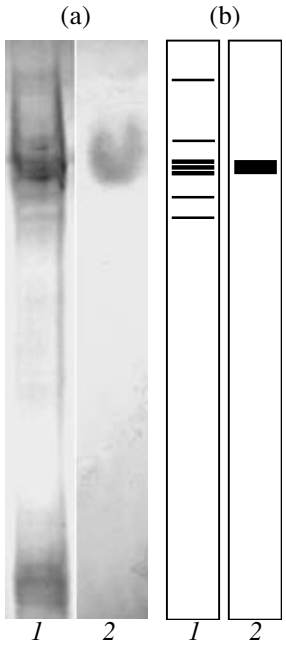

Fig. 2. Results of (*1*) SDS-PAGE and (*2*) western immunoblotting (rabbit ABLPS Cd) of the preparation of *A. brasilense* Cd LPS: a, original; b, schematic representation.

decanoic (3-OH-$C_{16:0}$), octadecenoic ($C_{18:1}$), and nanodecanoic ($C_{19:0}$) fatty acids were prevalent. Their ratio calculated as the ratio of percentages of peak areas (relative to the total area of peaks in the chromatogram) was 33.6 : 2.1 : 3.7 : 24.2 : 24.5 : 7.2. The fatty acid composition values of LPS_{Cd} are in a good agreement with data from the literature on LPSs of a variety of strains of *A. brasilense* [19, 20] and *A. lipoferum* [20]. Lipid A is known to be the most conservative part of the LPS macromolecule, and, for this reason, the similarity documented in this study may be indicative of the phylogenetic relationship between these two *Azospirillum* species, at least in the fatty-acid composition.

GLC of polyol acetates demonstrated the prevalence of rhamnose, mannose, and galactose in the preparation under study (ratio, 3 : 1 : 2). Determination of absolute configurations of the monosaccharides demonstrated that galactose and mannose have D-configurations, whereas rhamnose is present in the L-configuration. It is of note that both the monosaccharide composition and the ratio of neutral sugars of *A. brasilense* Cd OPS are similar to those of the OPSs of *A. lipoferum* Sp59b and *A. irakense* KBC1, in spite of the fact that the latter strains differ in the structure of oligosaccharide repeats [9, 10].

GLC–mass spectrometry of partially methylated polyol acetates demonstrated that the OPS of *A. brasilense* Cd contains residues of 2,4-di-O-methylrhamnose, 3,4-di-O-methylrhamnose, 2,4,6-tri-O-methylmannose, 2,4,6-tri-O-methylgalactose, 2,6-di-O-methylgalactose, and a terminal 2,3,4-tri-O-methylrhamnose. These data allowed us to conclude that the oligosaccharide repeat of the OPS is a branched hexasaccharide (containing a 3,4-substituted galactose in the branchpoint and a terminal rhamnose in the side chain), and that its constituent monosaccharides are represented by pyranose forms. Thus, analysis of the OPS of *A. brasilense* Cd by the method of methylation made it possible to identify in the reaction mixture the same products as in the OPS of *A. lipoferum* Sp59b [10]. This observation is indicative of the structural similarity of these polysaccharides.

Immunization of rabbits by chromatographically purified LPS_{Cd} elicited polyclonal antibodies, ABLP-S_{Cd}. The procedure used in this work proved to be highly efficient, judging by the observation that the concentration of specific antibodies in the serum was sufficient for pronounced precipitate formation in the double radial immunodiffusion test as early as after the second immunization.

The $ABLPS_{Cd}$ obtained was characterized by (1) the ability to cause agglutination of heat-inactivated homologous cells (the affinity for surface macromolecules was three times lower in encapsulated than capsule-free cells); (2) reactivity in immunodiffusion analysis with crude or deproteinized LPS; and (3) the capacity for binding only slowly migrating S-type LPS molecules in western immunoblotting (Fig. 2, track *2*).

Binding of the antibody to encapsulated cells is due to the presence of an LPS–protein complex (LPPC, an extracellular form of LPS) in the capsular material. The higher activity of the antibodies in binding capsule-free cells is apparently related to differences in the exposure

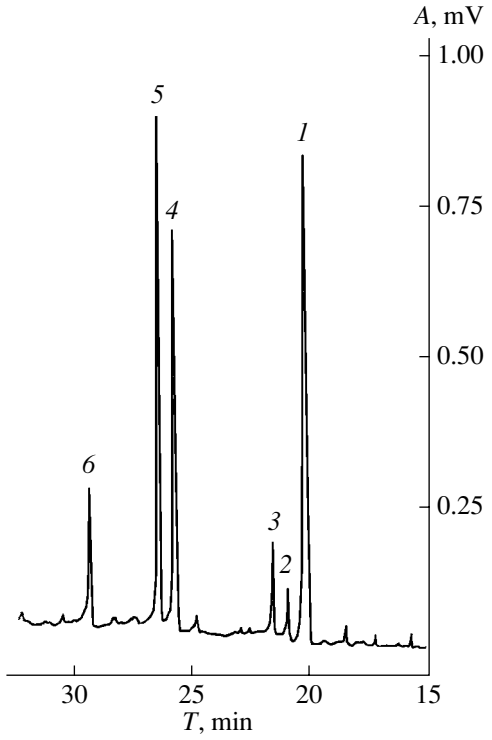

Fig. 3. GLC profile of methyl esters of fatty acids of lipid A of *A. brasilense* Cd LPS: *1*, 3-OH-$C_{14:0}$; *2*, $CC_{16:1}$; *3*, $C_{16:0}$; *4*, 3-OH-$C_{16:0}$; *5*, $C_{18:1}$; *6*, $C_{19:0}$. *T*, retention time; *A*, value proportional to methyl ester concentration in the sample.

of antigenic LPS determinants between the external membrane and the capsule. LPS is known to account for up to 70% of the surface area of the external membrane of gram-negative microorganisms. Based on the data obtained, we conclude that the capsular material of azospirilla (which comprises, in addition to LPC, a polysaccharide–lipid complex, free polysaccharides, and proteins) screens the O-antigen of the outer membrane; the determinants of extracellular LPS may be less accessible to the antibodies because of the formation of complexes with proteins.

Using the method of immunodiffusion and $ABLPS_{Cd}$, we were able to visualize no less than two antigenic determinants within LPS_{Cd} (Fig. 4, well *3*), the conformation of which did not change either in the course of prolonged boiling of the solution of LPS_{Cd} preparation or as a result of its treatment with proteinase K (a proteolytic enzyme). This demonstrates that the determinants were not proteinaceous. The observation that the antibodies exhibited identical activity with both the crude extract and the deproteinized LPS preparation is indicative of their high specificity. Comparison of the immunodiffusion patterns of LPS preparations isolated from diverse strains of azospirilla demonstrated that $ABLPS_{Cd}$ cross-reacted with LPSs of the closely related strain *A. brasilense* Sp7 (Fig. 4, well *2*) and the strain *A. lipoferum* Sp59b (Fig. 4, well *4*). Note that the cross-reaction with LPS_{Sp59b} was observed for

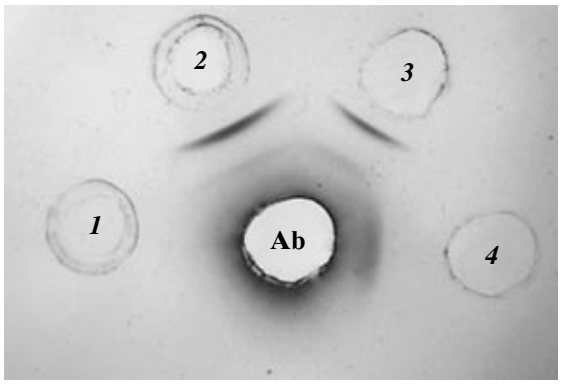

Fig. 4. Results of immunodiffusion analysis (with ABLPS Cd, Ab) of LPS preparations of *A. brasilense* Sp245 (*1*), *A. brasilense* Sp7 (*2*), *A. brasilense* Cd (*3*), and *A. lipoferum* Sp59b (*4*).

only one antigenic determinant of LPS_{Cd}. The results of ELISA (Fig. 5) demonstrated the antigenic similarity of strains *A. brasilense* Cd, *A. brasilense* Sp7, and *A. lipoferum* Sp59b, but not *A. brasilense* Sp245, which was almost completely dissimilar to its counterparts in this respect.

The antigenic cross-reactivity of LPSs of *A. brasilense* Sp7 and Cd may be due to the very close relationship between the two bacterial strains. *A. brasilense* Cd was isolated form the roots of *Cynodon dactylon* following inoculation of this plant with a culture of *A. brasilense* Sp7 [11]. It was demonstrated earlier that steady differences in the antigenic structure of LPSs of these strains, revealed by immunochemical methods, correlate with the loss of a 115-MDa plasmid by *A. brasilense* Cd [5].

The observation that $ABLPS_{Cd}$ react with the preparation of LPS_{Sp59b} is indicative of the existence of common antigenic determinants within LPSs of strains belonging to distinct species of the genus *Azospirillum*. Note that a unilateral reaction of *A. lipoferum* Sp59b antibodies with *A. brasilense* Sp7 LPS was documented in an earlier study [6] by immunodiffusion analysis with glutaraldehyde-treated whole cells of azospirilla. Our serological data agree with the results of rDNA sequence comparison, which demonstrated that *A. brasilense* and *A. lipoferum* exhibit maximum homology within the genus *Azospirillum* [21].

In conclusion, the macromolecular organization of LPS of *A. brasilense* Cd is common to the bacteria of this species. The preparation of this LPS was represented by the S-form of molecules. Analysis of the LPS demonstrated the presence of characteristic components, such as carbohydrates, hydroxylated fatty acids, KDO, and heptose. The fatty acid profile of lipid A was characteristic of typical representatives of the genus *Azospirillum*. The identical monosaccharide composition of LPSs of *A. brasilense* Cd and *A. lipoferum* Sp59b and their immunochemical cross-reactivity indicate that these <PSs have a similar portion as part of

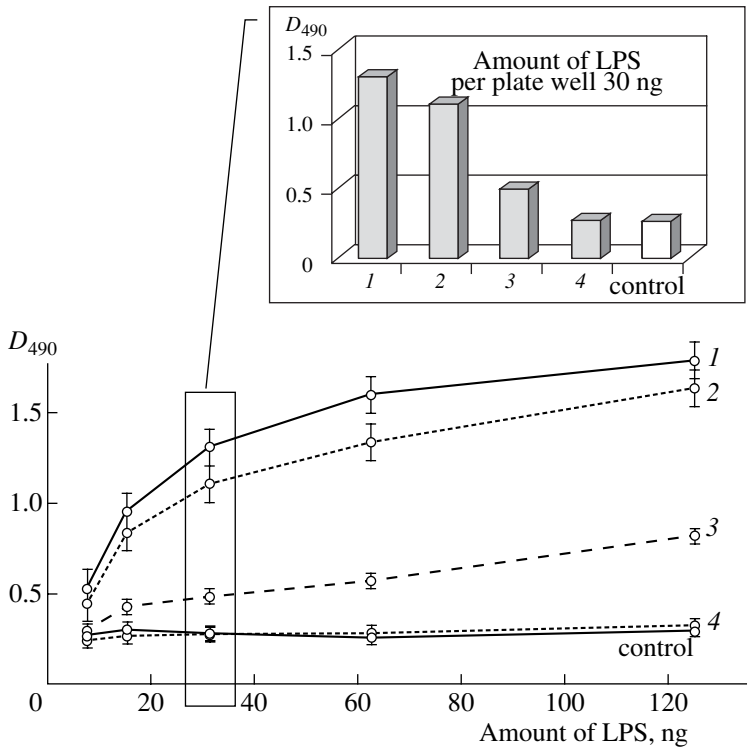

Fig. 5. Results of AB reactions with LPS preparations of *A. brasilense* Sp7 (*1*), *A. brasilense* Cd (*2*), *A. lipoferum* Sp59b (*3*), and *A. brasilense* Sp245 (*4*); the concentration of $ABLPS_{Cd}$ was equal to 35 μg/ml.

their OPS repeats and that this portion forms one of the immunodominant epitopes of LPS_{Cd}.

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