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EXPERIMENTAL ARTICLES

Study of Immunochemical Heterogeneity of *Azospirillum brasilense* Lipopolysaccharides

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Abstract—A comparative immunochemical analysis of lipopolysaccharides (LPS) in *Azospirillum brasilense* model strains Sp7 and Sp245 and in mutants with altered somatic antigens has been performed. According to the results of a complex of various immunochemical methods, including studies with polyclonal antibodies against the LPS these bacteria, their LPS consist of an assembly of macromolecules with different antigenic characteristics. Two types of O-specific polysaccharides (O-PS) are present in the LPS of every strain of *A. brasilense* under study. The major difference between the two O-PS is the antigenic heterogeneity of one of them. This heterogeneous O-PS has been shown to possess at least two O-factors (antigenic determinants) different in their structure. Meanwhile, according to all the tests performed, the other O-PS in every strain is immunochemically homogeneous and identical to one of the determinants revealed in the more diversified O-PS. The LPS heterogeneity among the given strains may be due to the pattern of O-specific polysaccharide synthesis, one of the O-PS being an intermediate in the synthesis of the other.

Key words: Azospirillum brasilense, lipopolysaccharides, antigenic determinants, immunochemical heterogeneity.

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One important issue in the study of bacterial somatic antigens is determination of their heterogeneity extent and the biological implication of this heterogeneity. The phenomenon of defects in the polysaccharide chains of lipopolysaccharides (LPS) or even their complete loss (in R–S dissociation) is well-known [1]. It was also shown that the O-specific polysaccharides (O-PS) synthesized by a given bacterial species can differ in structure [2–4].

According to the literature there are two types of bacterial LPS heterogeneity. First, O-PS molecules may vary in molecular mass and in their degree of branching with their monosaccharide composition staying the same. Denaturing PAG electrophoresis provides the best demonstration of these differences. Bacteria with marked heterogeneity of O-PS length form a characteristic "ladder" of bands where each "step" is a result of an increase in weight by one oligosaccharide unit [2, 5]. This type of heterogeneity is probably determined by the cultivation conditions. The O-PS chain length is known to vary depending on the culture growth phase; e.g., in *Yersinia pseudotuberculosis* the maximum chain length is achieved during the stationary phase, while the monosaccharide composition does not change depending on the growth phase [6]. The second type is chemical structure heterogeneity. In this case, the cells produce LPS with different chemical structures of the core and/or the polysaccharide chain. Sometimes LPS may consist of several different O-specific polysaccharides [3, 4, 7]. Thus, LPS are not an individual chemical compound, but a family of molecules varying in structure and molecular mass. The biological implication of this phenomenon may be a denser package of heterogeneous molecules on the cell surface.

Nitrogen-fixing bacteria of the genus *Azospirillum* influence the productivity of agricultural plants and thus are assigned to growth-stimulating rhizobacteria. The major component of the azospirilla cell surface is LPS or O-antigen, which represents an important determinant in the processes of molecular recognition between plants and these soil bacteria [8]; it is also the main criterion for the serological classification of azospirilla [9, 10].

The goal of the present work was to study in detail the immunochemical properties of heterogeneous LPS of the model *Azospirillum brasilense* strains Sp7 and Sp245.

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MATERIALS AND METHODS

The strains used in the study are listed in the table. The bacteria were grown to the late exponential growth phase at 30°C in a liquid synthetic malate medium [15] supplemented with NH_4Cl (1 g/l). Polyclonal strain-specific antibodies (Ab) against azospirilla LPS were obtained as described by Matora et al. [16]. The LPS extraction method described by Leive et al. [17] was used with modifications. Bacterial cells were washed with phosphate buffered saline (pH 7.2), centrifuged, and resuspended in an extraction buffer (pH 8.5) containing 0.1 M Tris–HCl, 10 mM EDTA, 0.1 mM PMSF, and 1% triton X-100 (EDTA concentration was 0.05 mM per 1 g of wet cells); extraction was carried out for 30 min at room temperature. The supernatant was separated from the cells by centrifugation.

The standard technique was used to perform double immunodiffusion. Agarose gels (1%) prepared on Tris–glycine–barbiturate buffer (ionic strength 0.02, pH 8.8) were used for immunophoresis. Linear electrophoresis was performed for 30 min at 10 V/cm. After electrophoresis, 100 μl of Ab (20 mg/ml) was introduced into each groove. For tandem crossed electrophoresis, the samples were injected into the first direction gel wells (without Ab). The gel plate was left for 30 min in a moist chamber to let the samples diffuse into the gel. The first direction electrophoresis was carried out for 30 min at 10 V/cm. The second direction gel contained 1% of the corresponding Ab and was run for 16 h at 2 V/cm. After the pressing and washing procedures, the gels were stained with Coomassie R-250.

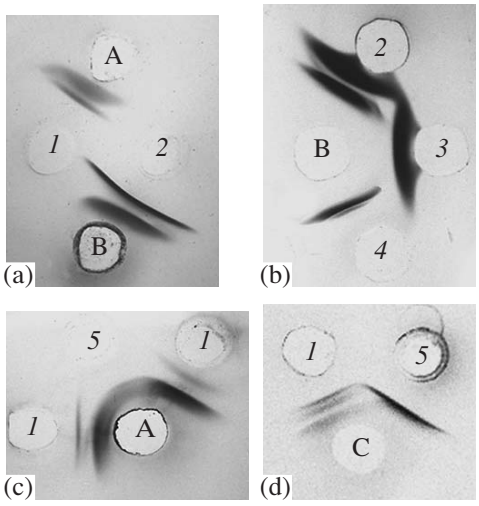

Fig. 1. Results of immunodiffusion analysis of the LPS preparations of *A. brasilense* strains Sp7 (*1*), Sp245 (*2*), KM018 (*3*), KM252 (*4*), and Sp7.K.2 (*5*) with Ab against LPS of (A) Sp7, (B) Sp245, and (C) Sp7.K.2. In the LPS of Sp7, S-O-PS (precipitation band closest to the well A) and R-O-PS (precipitation band closest to the well 1) are revealed. In the LPS of Sp245, O-PS1 (band closest to the well 2) and O-PS2 (band closest to the well B) are revealed.

Bacterial strains used in the work

A. brasilense strain	Characterization	Source
Sp7	Wild type	[11]
Cd	Wild type	[12]
Sp245	Wild type	[13]
Sp7.K.2	Spontaneous Sp7 mutant	Present work
KM018	Omegon Sp245 mutant with omegon-Km insertion in a 120-MDa plasmid	[14]
KM252	Omegon Sp245 mutant with omegon-Km insertion in a 120-MDa plasmid	[14]

The lipopolysaccharides were prepared for PAGE by treatment of the whole cells with proteinase as described by Hitchcock and Brown [5]. The gels were stained with silver for visualization [18].

RESULTS AND DISCUSSION

To study somatic antigen immunochemical heterogeneity of *A. brasilense*, we used two model strains, Sp7 and Sp245. Lipopolysaccharide preparations of these strains contain two O-PS each, forming two precipitation lines with homologous antibodies in the process of immunodiffusion (Fig. 1a). For comparative analysis of different LPS fractions, the mutant strains used were deficient in some of the O-specific polysaccharide.

The results of electrophoretic analysis of Sp7 LPS in comparison with the mutant variants of this strain with an altered LPS structure are presented in Fig. 2. Two

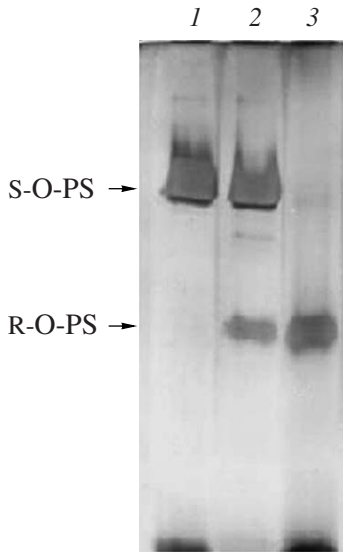

Fig. 2. *A. brasilense* LPS preparations PAGE results: Cd (*1*), Sp7 (*2*), and Sp7.K.2 (*3*).

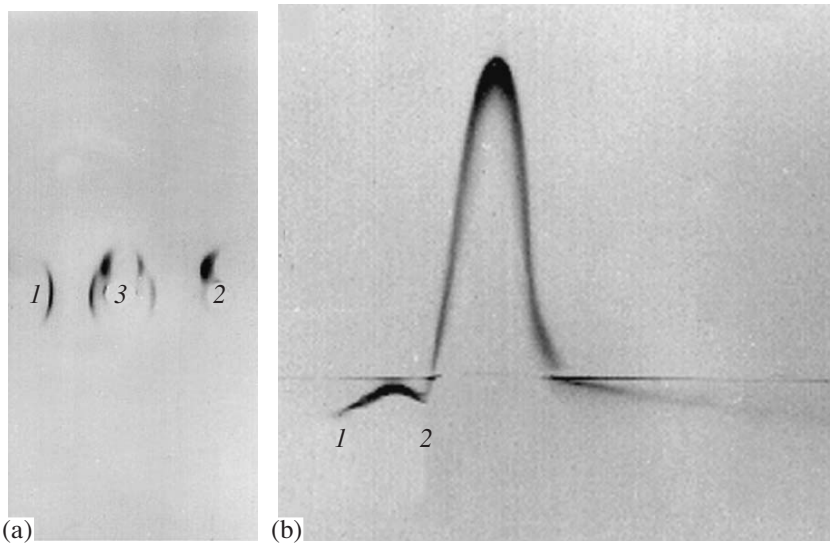

Fig. 3. Linear immunoelectrophoresis (a) and crossed-over tandem immunoelectrophoresis (b) of the LPS preparations of strains KM252 (*1*), KM018 (*2*), and Sp245 (*3*) with Ab against LPS of Sp245. See text for explanation.

bands are formed by two different O-PS characteristic of this strain, namely, R-O-PS and S-O-PS. The designations were introduced in our previous work [19], in which we have demonstrated that the R–S dissociation pattern in *A. brasilense* Sp7 type strain is determined by the relative contribution of these antigen structures to the cell surface composition.

In a comparative immunochemical study of the O-PS composing Sp7 LPS, we used the spontaneous mutant strain Sp7.K.2. It was selected from Sp7 after several freeze–thaw cycles of the type strain culture. This mutant and strain Cd [20] both lack a 115 mDa plasmid (data not presented). Electrophoresis of strain 7.K.2 LPS (Fig. 2), as well as immunodiffusion with the Ab against Sp7 LPS (Fig. 1c), detected only R-O-PS. Development of the antibodies recognizing LPS of Sp7.K.2 made it possible to evaluate the antigenic spectrum of R-O-PS. Fig. 1d demonstrates that a homogeneous precipitation band was formed in the immunodiffusion reaction between the Ab recognizing 7.K.2 and its antigen, while the reaction with the LPS of Sp7 type strain revealed the whole spectrum of bands characteristic of both R- and S-O-PS. Hence, R-O-PS contains all the antigenic determinants characteristic of the wild type Sp7 strain, including those of S-O-PS. In our earlier work [20], the Ab against the LPS of strain Cd (cf. the appropriate electrophoregram in Fig. 2) have been shown to recognize only S-O-PS of the type strain. These facts suggest that R-O-PS of strain Sp7, unlike S-O-PS, is an immunochemically heterogeneous LPS, probably a product of some precursor.

Another *A. brasilense* strain, Sp245, demonstrates a similar pattern of immunochemical heterogeneity of O-specific polysaccharides. Two O-PS of strain Sp245 were previously designated as O-PS1 and O-PS2. Omegon mutants of Sp245, KM018 and KM252, deficient in synthesis of either O-PS1 or O-PS2, were obtained and characterized earlier [14]. In the present research these mutant strains were used for immunochemical comparison of two O-PS.

In the immunodiffusion test (Fig. 1b), the Ab against the LPS of the original strain were bound by O-PS1 in KM018 LPS preparation and by O-PS2 in the LPS of KM252, respectively. According to immunoelectrophoresis (Fig. 3a), O-PS1 and O-PS2 differ in charge, the former being an acidic polysaccharide exhibiting anodic motility and the latter being neutral. The results of the chemical analysis carried out by Fedonenko et al. [21] confirmed the occurrence of two types of O-PS in the type strain, while lipopolysaccharides of the mutant strain KM252 contain only neutral O-PS and those of KM018 contain only the acidic one.

The merging bands of immunoprecipitation in tandem crossed immunoelectrophoresis (Fig. 3b) provided evidence of the common antigenic determinants in O-PS1 and O-PS2. The picture of immunoelectrophoresis clearly revealed a spur, a one-side extension of the O-PS2 precipitation line. The spur indicates that O-PS2 contains antigenic determinants not present in O-PS1.

It should be mentioned that the two O-PS of *A. brasilense* Sp245 are not only dissimilar from the immunochemical point of view, but also differ in their ability to modify their determinants depending upon environmental factors. For example, O-PS1, unlike O-PS2, was capable of antigen modification caused by the presence of Tris cations in the medium [22].

Unlike immunochemical tests, acidic hydrolysis of O-PS1 and O-PS2 did not reveal any differences between them. The IR and NMR spectra of these polysaccharides were identical and proved the given

O-PS to be homopolymers of D-rhamnan [21]. Our results confirm the concept that the serological specificity of O-antigens is mainly determined by the antigen determinants of O-PS (so-called O-factors), rather than the major polysaccharides [23]. Consequently, the main difference between O-PS1 and O-PS2 is probably the antigenic heterogeneity of the former caused by the presence of at least two structurally different antigenic determinants.

To sum up the results of comparative immunochemical study of the LPS of different *A. brasilense* model strains, we should note the following. At least two structurally different O-factors (antigenic determinants) are present in one of the two O-PS, found in the LPS of model strains Sp7 and Sp245, while another O-PS is immunochemically homogeneous and identical to one of the O-factors present in the heterogeneous O-PS. Thus, we suggest that the homogeneous O-specific polysaccharide of these strains is a precursor of the heterogeneous one.

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