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EXPERIMENTAL ARTICLES

Atypical R–S Dissociation in *Azospirillum brasilense*

L. Yu. Matora[1], O. B. Serebrennikova, L. P. Petrova, G. L. Burygin, and S. Yu. Shchegolev

Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, pr. Entuziastov 13, Saratov, 410049 Russia

[1]E-mail: larisa@ibppm.sgu.ru

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Abstract—It was found that atypical R–S dissociation in the type strain *A. brasilense* Sp7 is not accompanied by drastic changes in the polysaccharide moieties of bacterial lipopolysaccharides but is rather due to different contributions of two O-specific polysaccharides (found in both R and S dissociants) to the age-dependent architectonics of the cell surface.

Key words: *Azospirillum brasilense* Sp7, R–S dissociation, lipopolysaccharides, monospecific antibodies.

R–S dissociation in bacteria is a manifestation of their mutational variability. It is considered that R and S bacterial forms differ in that R-type cells contain lipopolysaccharides lacking, partially or completely, an O-specific polysaccharide [1].

Bacteria of the genus *Azospirillum* are gram-negative plant growth–promoting diazotrophs [2]. The spontaneous morphological variability of colonies of the *A. brasilense* type strain Sp7 was described in a number of publications [3–5]. In particular, Matveev *et al.* showed that S-type cells of strain Sp7 differ from wild-type R cells in that the former cells are unable to synthesize orange pigment and to absorb the dye Congo Red and lack the 115-MDa plasmid p115 in the plasmid profile [3]. Later, it was shown that the R–S dissociation of strain Sp7 is not related to changes in the lipopolysaccharide components of the outer membrane of cells [6].

Katupitiya *et al.* described a mutant of *A. brasilense* Sp7, which was phenotypically very close to the S variant of this strain, was unable to absorb Congo Red, did not flocculate, and contained no material that was found on the surface of the wild-type Sp7 cells [5]. That mutant was presumably defective in exopolysaccharide synthesis.

The little knowledge of structural changes in *Azospirillum* cells related to their R–S dissociation prompted us to comparatively study the antigenic properties of the R and S dissociants of *A. brasilense* Sp7.

MATERIALS AND METHODS

The wild-type strain *A. brasilense* Sp7 was obtained from the LMG Culture Collection (Belgium). Sp7-S is a spontaneous mutant of strain Sp7, which is formed at a frequency of 10^{-1} [3]. Unlike S-type cells, R-type cells have the wild-type phenotype. Arbitrarily, the latter kind of cells is called strain Sp7-R. The strains were grown at 30°C in a liquid synthetic medium with malate [2] and 1 g/l NH_4Cl for 18 h or on the same medium solidified with 1.5% agar for 72 h. The spontaneous variant Sp7-S differed from the wild-type variant Sp7-R in that it does not contain plasmid p115 in the plasmid profile.

The immunoelectrophoretic and immunodiffusion analyses of R and S antigens in the LPSs from R- and S-type cells of *A. brasilense* Sp7

LPSs from	Antibodies to LPSs from			
	18-h-old R cells	72-h-old R cells	18-h-old S cells	72-h-old S cells
18-h-old R cells	RS	–R	–S	RS
72-h-old R cells	RS	–R	–S	RS
18-h-old S cells	RS	–R	–S	RS
72-h-old S cells	RS	–R	–S	RS

Note: "R" and "S" indicate the presence of the precipitation lines of the corresponding antigen. "–R" and "–S" indicate the absence of the precipitation lines of R and S antigens, respectively.

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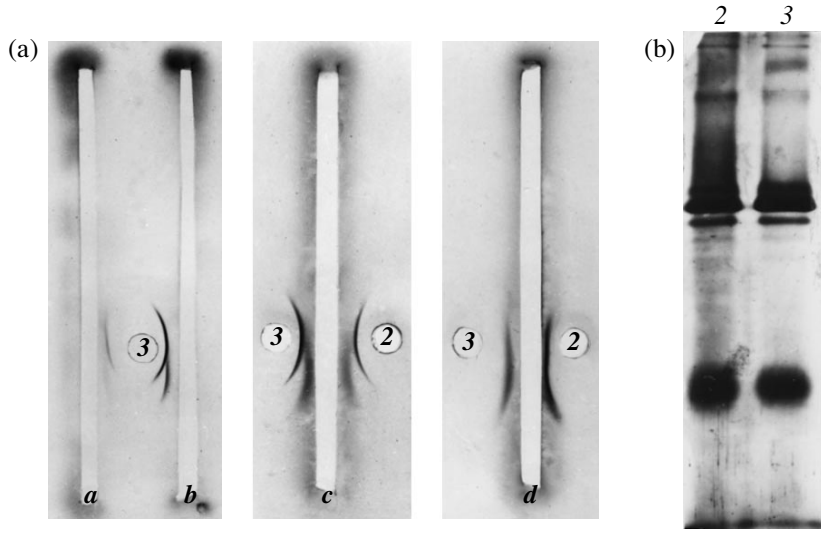

Fig. 1. (a) Linear immunoelectrophoresis of the LPSs isolated from (*2*) 18-h-old R-type cells and (*3*) 18-h-old S-type cells with antibodies to the LPSs of (*a*) 18-h-old S-type cells, (*b*) 72-h-old S-type cells, (*c*) 18-h-old R-type cells, and (*d*) 72-h-old R-type cells; (b) the PAAG electrophoresis of the LPSs isolated from (*2*) 18-h-old R-type cells and (*3*) 18-h-old S-type cells.

The plasmid profile of cells was determined as described by Eckhardt [7].

Monospecific antibodies to lipopolysaccharides (LPSs) were obtained using glutaraldehyde-treated intact *A. brasilense* cells for immunization [8].

LPSs were isolated as described by Leive *et al.* [9].

Double immunodiffusion analysis and linear immunoelectrophoresis were carried out by the standard procedures [10, 11].

LPS chemotypes were determined by the PAAG electrophoresis of the LPSs obtained by the proteinase treatment of whole cells [12]. Separated LPSs were visualized by silver staining [13] or transferred by electroelution onto 0.45-μm nitrocellulose Millipore filters. Immunodetection was performed by incubating blots with the LPS antibodies (50 μg/ml), antirabbit antibodies conjugated with horseradish peroxidase (Sigma), and 3,3'-diaminobenzidine [14].

Possible changes in the monosaccharide composition of LPSs were tested by recording the absorption spectra of their reaction products with phenol and sulfuric acid in the wavelength interval 400–520 nm [15].

RESULTS AND DISCUSSION

The 18-h-old colonies of strains Sp7-R and Sp7-S were smooth and glossy and did not differ from each other. At the same time, the 72-h-old colonies of the wild-type strain Sp7-R had a rough surface and uneven edges, while the colonies of the mutant strain Sp7-S of the same age remained smooth and glossy and did not have phenotypic traits typical of strain Sp7-R.

This prompted us to investigate the dynamics of LPSs in these two strains as a function of age. For this purpose, we obtained monospecific antibodies to the LPSs of 18- and 72-h-old R- and S-type cells using glutaraldehyde-treated intact cells for immunization [8]. It was found that antibodies to 18-h-old R-type cells produced two precipitation lines with the LPSs isolated from these cells, whereas antibodies to 72-h-old R-type cells produced one precipitation line with the LPSs isolated from these cells (Fig. 1a, samples *c*, *d*). In contrast, antibodies to 18-h-old S-type cells produced one precipitation line with the LPSs isolated from these cells (Fig. 1a, sample *a*), and antibodies to 72-h-old S-type cells produced two precipitation lines with the

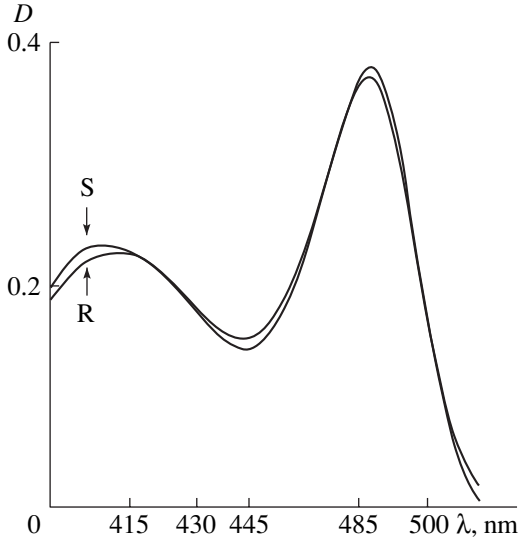

Fig. 2. The absorption spectra of the reaction products of the LPSs isolated from 18-h-old R cells (R) and 18-h-old S cells (S) with phenol and sulfuric acid.

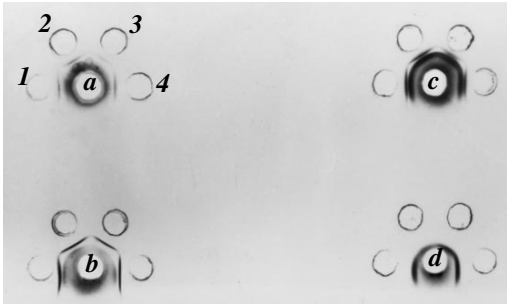

Fig. 3. Comparative immunodiffusion analysis of the LPSs isolated from (*1*) 72-h-old S cells, (*2*) 18-h-old S cells, (*3*) 18-h-old R cells, and (*4*) 72-h-old R cells with antibodies to the LPSs isolated from (*a*) 18-h-old S cells, (*b*) 72-h-old S cells, (*c*) 18-h-old R cells, and (*d*) 72-h-old R cells.

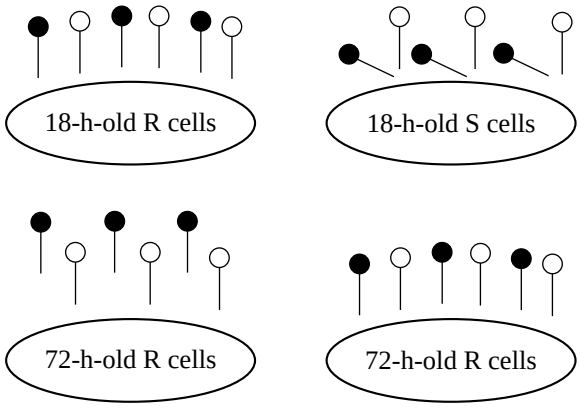

Fig. 4. Diagrams illustrating the putative distribution of O-specific antigens upon the R–S dissociation of *A. brasilense* Sp7. Dark and open circles represent R and S antigens, respectively.

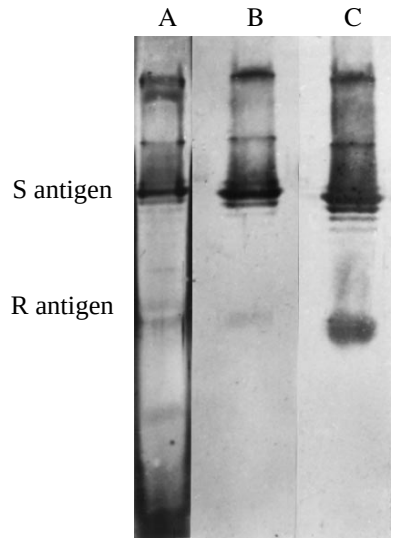

Fig. 5. (A) The PAAG electrophoresis of the LPSs isolated from 18-h-old R-type cells and the visualization of S and R antigens in the immunoblot obtained with antibodies to (B) 18-h-old and (C) 72-h-old S-type cells.

LPSs isolated from these cells (Fig. 1a, sample *b*). At the same time, the LPSs of 18-h-old R-type and S-type cells were identical in the number of antigens (two) (Fig. 1a), were very close in their electrophoretic profiles in PAAG (Fig. 1b), and had similar absorption spectra of their reaction products with phenol and sulfuric acid (Fig. 2). According to Dubois *et al.* [15], the similarity of such spectra for preparations tested implies that their monosugar compositions are close.

Figure 3 shows the results of the immunodiffusion analysis of four preparations of LPSs with four kinds of antibodies to them. As can be seen from this figure, the immunoprecipitation pattern depends on the kind of antibodies and does not depend on the variant and the age of the culture from which antigens (LPSs) were isolated.

The above data, which are summarized in the table, allow the suggestion to be made that the R–S dissociation of *A. brasilense* Sp7 involves altered synthesis of one O-specific polysaccharide, which was arbitrarily designated as R antigen. The other of the two detected O-specific polysaccharides was designated as S antigen. As can be inferred from the diagrams presented in Fig. 4, the predominant synthesis of R antigen at the late growth stages of strain Sp7-R and its masking of S antigen may explain the specific morphocolonial and immunological properties of this strain. In 18-h-old S-type cells, R antigen is masked by S antigen. However, in 72-h-old S-type cells, R antigen is exposed and may exert its immunogenic action.

Using ELISA with antibodies to the LPSs of S-type cells, we visualized R and S antigens in the LPSs of strain Sp7 (Fig. 5). As can be seen from this figure, antibodies to the LPSs of 18-h-old S-type cells clearly reveal S antigen and virtually no R antigen. At the same time, antibodies to the LPSs of 72-h-old S-type cells reveal both S and R antigens because R antigen in old S-type cells becomes immunogenically equal to S antigen.

Thus, the atypical R–S dissociation of *A. brasilense* Sp7 is presumably due to the different contributions of two O-specific polysaccharides of this strain, arbitrarily designated as R and S antigens, to the age-dependent architectonics of the bacterial cell surface.

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