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EXPERIMENTAL ARTICLES

Characterization of Lipopolysaccharides from *Ralstonia solanacearum*

L. D. Varbanets, V. N. Vasil'ev, and O. S. Brovarskaya

Zabolotnyi Institute of Microbiology and Virology, National Academy of Sciences of Ukraine, ul. Zabolotnogo 154, Kiev, 252143 Ukraine

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Abstract—Lipopolysaccharides (LPSs) from four strains of *Ralstonia solanacearum* belonging to biovar I (ICMP 6524, 8115, 5712, and 8169) were isolated and investigated. The structural components of the LPS molecule, such as lipid A, the core oligosaccharide, and O-specific polysaccharide (O-PS), were obtained after mild acid hydrolysis of the LPS preparations. In lipid A from all the LPS samples studied, 3-hydroxytetradecanoic, 2-hydroxyhexadecanoic, tetradecanoic, and hexadecanoic fatty acids prevailed. The dominant monosaccharides of the core oligosaccharides of all of the strains studied were rhamnose, glucose, glucosamine, 2-keto-3-deoxyoctulosonic acid, and heptose. However, individual strains varied in the content of galactose, ribose, xylose, and arabinose. Three types of the O-PS structure were established, which differed in their configuration (α or β), as well as in the type of the bond between glucosamine and rhamnose residues (1 $\longrightarrow$ 2 or 1 $\longrightarrow$ 3).

Key words: Ralstonia solanacearum, lipopolysaccharide.

The species *Ralstonia solanacearum* is heterogeneous with respect to biological and biochemical properties of individual strains. The systematics of this species is not yet clear; its representatives have long been assigned to the genus *Pseudomonas*. Thus, according to the ninth edition of *Bergey's Manual* [1], the species *R. solanacearum* is assigned to the RNA homology group II, which also includes the species *P. mallei*, *P. solanacearum*, *P. caryophylii*, *P. cepacia*, *P. gladioli*, and *P. pickettii*, differing in their physiological properties. Yabuuchi *et al.* [2] investigated representatives of RNA homology group II of the genus *Pseudomonas* with the aim of revising their taxonomic position. On the basis of the results of 16S rRNA sequence analysis, DNA–DNA hybridization, composition of cellular lipids and fatty acids, and phenotypic characteristics of the strains studied, the authors suggested the transfer of the representatives of the RNA homology group II from the genus *Pseudomonas* to a new genus *Burkholderia*, with the type species *B. cepacia*; this transfer has been validated [3]. However, *B. solanacearum* and *B. pickettii* were distinct from other species of the genus *Burkholderia* in their properties; e.g., their lipids did not include ornithine-containing lipids (OL-1 and OL-2), whose presence is considered to be a characteristic feature of the genus *Burkholderia* (they are abundant in other species of this genus). Moreover, strains of *B. solanacearum* and *B. pickettii* showed a low degree of DNA–DNA homology with *P. aeruginosa* (10%) and five species of the genus *Burkholderia* (from 9 to 14%). Later studies provided further evidence for distinguishing the species *B. solanacearum* and *B. pickettii* from other species of the genus *Burkholderia* [4]. A new genus, *Ralstonia* (named in honor of the author who described the species *P. pickettii*), was established [4]; this genus included the species *B. solanacearum*, *B. pickettii*, and also *Alcaligenes eutrophus*, which was shown to be closely related to the first two species. Most likely, this classification is not final, since the heterogeneity of *R. solanacearum* representatives dictates the necessity of further investigation of their taxonomic position.

Since biochemical, structural, and functional peculiarities of lipopolysaccharides (LPSs) are considered to be an important chemotaxonomic criterion and may be applied to clarify the intraspecific classification of *R. solanacearum* and its interrelations with other species, the aim of this work was to study the composition and structural features of the LPS from *R. solanacearum*.

MATERIALS AND METHODS

The study was carried out with four strains of *Ralstonia solanacearum* obtained from the ICMP collection (ICMP 5712, 8169, 6524, and 8115). The cultures were grown in synthetic medium [5] on a shaker (240 rpm) at 28°C for 36–40 h; the cells were sedimented by centrifugation and dehydrated by treatment with acetone and diethyl ether. The LPS was extracted from dry biomass with a water–phenol mixture [6]; the water layer was dialyzed sequentially against tap and distilled water. Nucleic acids were removed either by precipitation with trichloroacetic acid or by ultracen-

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Table 1. Composition of monosaccharides of the LPSs from *R. solanacearum*

Strains	Preparations	Monosaccharides									
		% of the total of peak areas							% of dry mass		
		rhamnose	fucose	glucose	galactose	ribose	xylose	arabinose	glucosamine	KDO	heptose
5712	LPS	61.6	–	10.6	–	10.9	7.9	9.0	8.25	0.20	2.29
	O-PS	92.6	–	3.4	–	–	4.0	–	8.05	0.41	–
	Core oligosaccharide	71.4	–	20.3	–	4.4	3.9	–	3.4	1.9	9.24
6524	LPS	80.0	14.0	2.2	3.0	–	0.8	–	5.0	–	0.5
	O-PS	90.0	–	9.0	–	–	1.0	–	10.0	0.4	0.8
	Core oligosaccharide	59.0	25.0	7.2	6.8	–	–	2.0	6.0	2.73	3.0
8115	LPS	84.0	–	9.6	–	2.4	4.0	–	16.4	–	2.4
	O-PS	92.0	–	2.7	–	–	–	5.3	19.7	0.41	3.6
	Core oligosaccharide	45.2	–	45.7	–	5.2	3.9	–	2.0	5.3	8.6
8169	LPS	70.5	–	12.9	–	6.7	9.9	–	9.4	0.26	5.8
	O-PS	86.8	–	2.1	–	–	11.1	–	10.05	0.50	7.8
	Core oligosaccharide	76.4	–	16.9	Tr.	2.5	4.2	–	4.4	3.2	16.4

Note: "–" stands for "not detected."

trifugation of the supernatant; the purified LPS was lyophilized.

The structural components of the LPS macromolecule, O-specific polysaccharide (O-SP), core oligosaccharide, and lipid A, were isolated by hydrolysis of the preparation with 1% acetic acid (100°C, 2 h) after preliminary removal of phospholipid contaminations by extraction with methanol and chloroform. The sediment of lipid A was collected by ultracentrifugation (25000 *g*, 40 min); the supernatant was concentrated to approximately 10 ml and fractionated on a column (70 × 3 cm) with Sephadex G-50 using 0.025 M pyridine–acetate buffer (pH 4.5) as the eluting agent.

The LPS fraction was analyzed by earlier described methods for the contents of reducing substances, heptoses, 2-keto-3-deoxyoctulosonic acid (KDO) [6], protein [7], and nucleic acids [8].

To determine the monosaccharide composition of the LPS, the preparations were hydrolyzed with 2 N HCl (100°C, 5 h); the obtained acetate polyols were analyzed by GLC on a Chrom-5 chromatograph (Czech Republic) equipped with a flame ionization detector and a column (3.0 mm × 1.2 m) packed with 3% neopentyl glycol succinate on Chromosorb W (80–100 mesh); a programmed temperature range of 170–200°C was scanned at 5°C/min.

Amino acids and hexosamine released after the hydrolysis of LPS preparations with 6 N HCl (100°C, 20 h) were assayed on a KLA-5 amino acid analyzer (Hitachi, Japan).

To determine the fatty acid composition of the LPS, the preparations were hydrolyzed with a 1.5% solution of acetyl chloride in methanol (100°C, 4 h) in sealed ampoules and analyzed on a GC 3400 gas chromatograph (Varian) combined with an ITD 800 mass spectrometer (Finnigan, Germany) and an IBM PC AT computer; a quartz capillary column (30 m × 0.33 mm) packed with OV-1701 (0.25 μm) was used; helium was the carrier gas (flow rate, 50 cm/s). The temperature program started at 125°C for 1 min and then increased at 7°C/min to 275°C, where it was held for 10 min. Operation parameters of the ITD-800 ion trap were the following: range of registered masses, 35–400 Da; scanning rate, 1 scan/2 s; microscanning frequency, 10 scans/s; multiplier voltage, 1900 V. The sensitivity of the method was 50 pg in the sample analyzed.

Identification of fatty acids was performed using a computer database of standard spectra from the National Bureau of Standards of the United States, which contains 42000 mass spectra of various compounds. Internal calibration was performed for quantitative determination of fatty acids.

RESULTS AND DISCUSSION

The diversity of biological properties of *R. solanacearum* strains makes it possible to divide them into

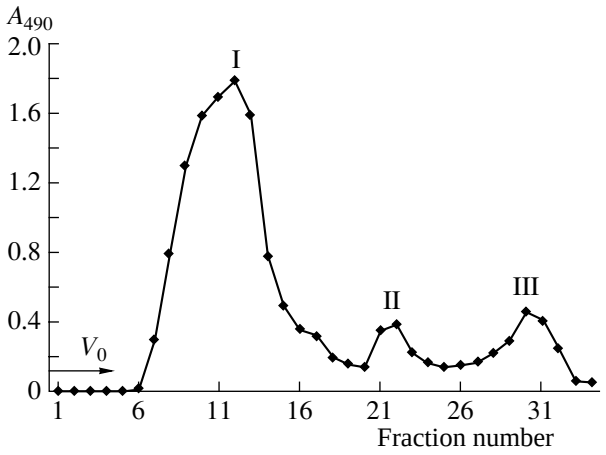

Fig. 1. Elution profile from a Sephadex G-50 column of the carbohydrate moiety of a hydrolyzed LPS from *R. solanacearum:* (I) the fraction of O-specific polysaccharide; (II, III) the fractions of the core oligosaccharide; (V_0) dead volume of the column.

races, biovars, and pathovars based on the type of colonies, phage specificity, and other properties. For example, strains of *R. solanacearum* isolated from all over the world were grouped into four biovars [9, 10], of which biovar I was the most heterogeneous [10]. We studied four strains of *R. solanacearum* belonging to biovar I, which were isolated from tomato (ICMP 5712, 8115, and 8169) and tobacco (ICMP 6524) plants.

The LPS fraction extracted from *R. solanacearum* cells with a water–phenol mixture contained a rather large amount of nucleic acids (up to 35%), which could be a result of the isolation procedure applied; however, high contamination of the LPS fractions by nucleic acids appears to be typical of *R. solanacearum* representatives [11]. The removal of nucleic acids from the LPS preparations was carried out by repeated ultracentrifugation, which reduced their content to 1.0–1.5%. However, in the case of the LPS fraction from *R. solanacearum* 6524, this method was improper: the content of nucleic acids remained as high as 13.5%. Therefore, the LPS fraction from this strain was purified by its treatment with a saturated solution of trichloroacetic acid, which forms insoluble complexes with nucleic acids; as a result of this purification, the content of nucleic acids was reduced to 0.5–2.0%, although the loss of LPS was higher than in the case of the ultracentrifugation procedure because of partial precipitation of the LPS together with the nucleic acids.

As can be seen from Table 1, the LPS fractions from all of the strains of *R. solanacearum* studied contained, after their purification from nucleic acids, rhamnose as the predominant (61.6–84.0%) monosaccharide (this is typical of *Pseudomonas* and *Burkholderia* species [12]), glucose (2.2–12.9%), and xylose (0.8–9.9%). The LPSs from *R. solanacearum* strains 5712, 8115, and 8169 contained 10.9, 2.4, and 6.7% ribose. Since the content of nucleic acids in the LPS preparations did not exceed 1.0–1.5%, the observed amounts of ribose cannot be impurities derived from nucleic acids. Recently, ribose was revealed in the LPS from *Escherichia coli* [13]. As can be seen from Table 1, the LPSs from *R. solanacearum* strains contained glucosamine (8.25–16.4 %) and heptose (0.5–5.8%), whereas KDO was revealed in the native LPS fractions in insignificant amounts (0.20–0.26%) or could not be detected at all. According to present-day knowledge, all the bacterial LPSs, regardless of their origin, contain at least one KDO residue or its derivative; bacterial cells with impaired KDO synthesis are not viable. However, revealing of KDO in the LPS preparations is often troublesome because of the possible substitution of its functional groups with certain residues hampering its identification. For instance, in the event that the KDO molecule contains a phosphate group at the 4th (or 5th) carbon atom and a carbohydrate chain at the 5th (or 7th) position, the formation of the OHC–CH_2–CO–COOH fragment, which gives a positive reaction with thiobarbituric acid, is impossible [14].

Thus, we revealed that the LPS from *R. solanacearum* contained all components typical of this biopolymer.

To isolate individual structural components of the LPS molecule, we applied mild acid hydrolysis, which breaks the bond between the KDO residue in the core oligosaccharide and the glucosamine (II) residue in lipid A. Lipid A was collected by centrifugation of the hydrolysate; water-soluble components were fractionated on a column with Sephadex G-50. In all of the strains studied, native LPS preparations contained a mixture of the S and R types of molecules, as evidenced by the occurrence of the high-molecular-weight fraction of O-specific polysaccharide (O-PS) (fraction I) and low-molecular-weight fractions of the core oligosaccharide (fractions II and III) (Fig. 1).

Lipid A is the most evolutionarily conservative moiety of the LPS molecule; it is built up of a 1,4'-diphosphorylated β-1,6-linked diglucosamine residue and four residues of (R)-3-hydroxy fatty acids bound via amide or ester bonds and carrying two or four nonhydroxylated acyl groups, respectively. The lipid A fractions from various bacterial species differ in the composition of fatty acids (first of all, of hydroxy acids), which is considered to be a stable characteristic and may serve as a chemotaxonomic criterion.

Lipid A from all the *R. solanacearum* strains studied contained even-numbered fatty acids (from C_{12} to C_{20}), with the predominance of 3-hydroxytetradecanoic, tetradecanoic, and 2-hydroxyhexadecanoic acids (Figs. 2a–2d, Table 2). Strain *R. solanacearum* 8115 was characterized by a high content of eicosadienoic ($C_{20:2}$) acid, which is extremely rare in bacteria. Lipids A of the LPSs from the strains studied also contained dodecanoic, hexadecanoic, and octadecanoic acids, which commonly occur in lipids A from bacteria

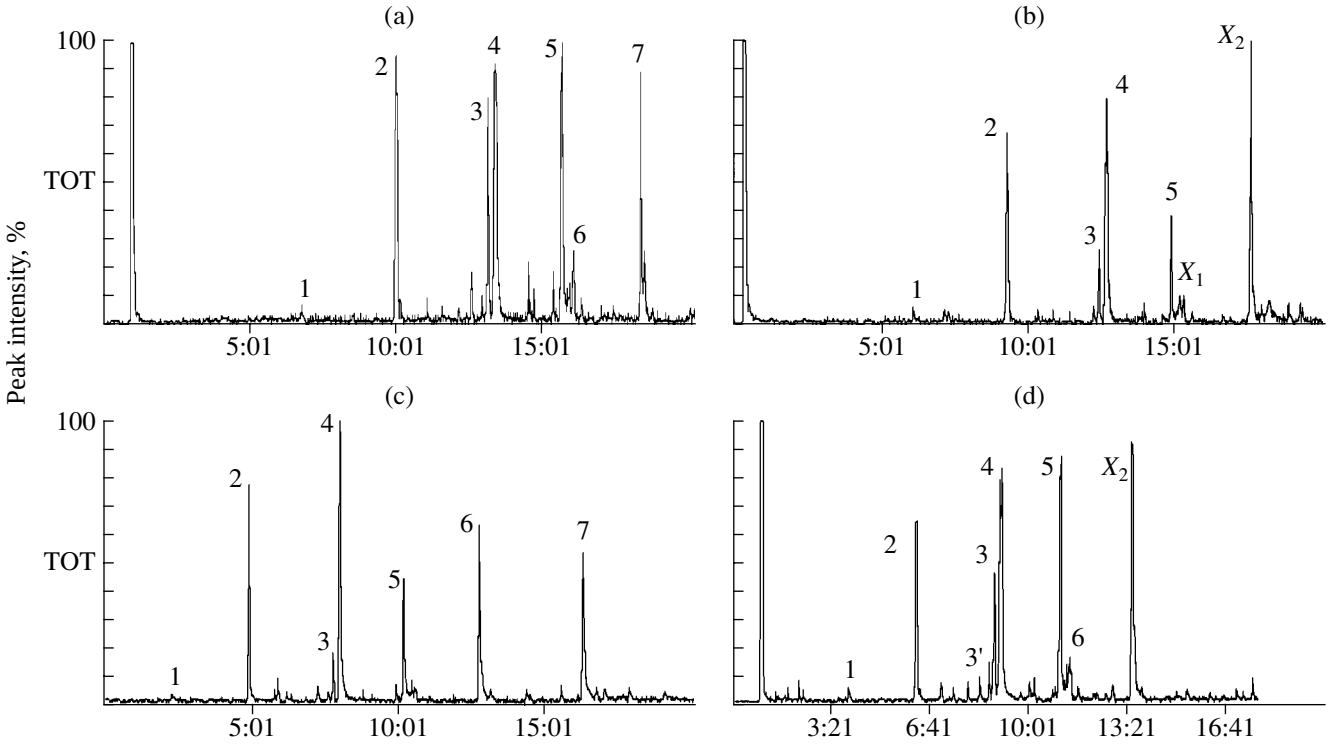

Fig. 2. Fatty acid composition of lipid A of the LPSs from *R. solanacearum* strains: (a) 5712; (b) 6524; (c) 8115; (d) 8169. (1) $C_{12:0}$; (2) $C_{14:0;}$ (3′) $C_{16:1}$; (3) $C_{16:0}$; (4) 3-OH-$C_{14:0}$; (5) 2-OH-$C_{16:0}$; (6) $C_{18:0}$; (7) $C_{20:2}$; (X_1); (X_2) unidentified acids.

belonging to section I of the genus *Pseudomonas*. Unsaturated fatty acids revealed in lipid A from *R. solanacearum* were $C_{16:1}$ in strain 8169 and $C_{20:2}$ in strains 8115 and 5712. Unsaturated fatty acids are not common components of lipid A, although they have been revealed in the composition in a few cases [15].

As can be seen from Fig. 2 and Table 2, the strains of *R. solanacearum* under study were characterized by a high content of hydroxy acids. It is believed that the profile of 3-hydroxy acids can serve as an additional chemotaxonomic criterion for the elucidation of the phylogenetic relationship between microorganisms [16]. Thus, lipids A from all enterobacteria contain only 3-OH-$C_{14:0}$ acid, which acylates both amino and hydroxy groups of glucosamine residues; *N*-linked 3-OH-$C_{14:0}$ and O-linked 3-OH-$C_{12:0}$ acids are typical of the family *Vibrionaceae*. Lipids A of pseudomonads contain 3-OH-$C_{12:0}$ and 3-OH-$C_{10:0}$ acids (rRNA homology group I), 3-OH-$C_{14:0}$ and 3-OH-$C_{16:0}$ acids (group II), or 3-OH-$C_{10:0}$ acid only (group III). However, this correlation was not always observed; for instance, certain strains belonging to the species *P. syringae* lack either 3-OH-$C_{10:0}$ or 2-OH-$C_{12:0}$ acids. Distinctions in the composition of fatty acids were also observed between individual strains belonging to the species *B. cepacia* and *R. solanacearum* [16]. It should be noted that the studies of lipids A from *R. solanacearum* strains are scarce; investigations were performed with cell hydrolysates rather than with the lipid A fractions. Since *R. solanacearum* is a heterogeneous species, whose systematics remains subject to debate, some doubts are always cast on whether a true representative of this species has been investigated.

From the predominance of 3-hydroxytetradecanoic and 2-hydroxyhexadecanoic acids, as well as tetra- and hexadecanoic acids, in lipids A of the LPSs from all four strains of *R. solanacearum* studied, it can be inferred that they are intrinsic components of the LPS from *R. solanacearum*, differentiating it from other bacterial species.

Analysis of our results obtained and literature data shows, on the one hand, that the present taxonomic classification of microorganisms is imperfect and, on the other hand, that fatty acid composition of lipid A cannot be a universal chemotaxonomic criterion; one of the reasons for this is its dependence on the methods of lipid hydrolysis and identification of fatty acids. For example, it has been reported that unsaturated fatty acids may be artifacts formed during degradation of 3-hydroxy acids [15].

The core oligosaccharide is characterized by a more variable composition than lipid A. The dominant components of the core oligosaccharide from the *R. solanacearum* strains studied were rhamnose and glucose, which, together with heptose and KDO, are typical of bacteria belonging to the family *Pseudomonadaceae*; the core oligosaccharides also contained such rare components as galactose (from trace amounts to 6.8%),

Table 2. Fatty acid composition of the lipids A of the *R. solanacearum* LPSs

Strains	Fatty acids (% of the total fatty acids)									
	$C_{12:0}$	$C_{14:0}$	$C_{16:1}$	$C_{16:0}$	3-OH-$C_{14:0}$	2-OH-$C_{16:0}$	$C_{18:0}$	$C_{20:2}$	X_1	X_2
5712	0.5	21.0	–	11.0	31.3	24.2	3.0	9.0	–	–
6524	0.5	18.2	–	3.5	33.7	10.8	–	–	17.6	15.7
8115	1.1	17.7	–	5.5	35.5	6.8	1.6	31.8	–	–
8169	0.9	15.5	1.7	8.6	27.9	19.5	2.4	–	23.5	–

Note: "–" stands for "not detected."

ribose (from 2.5 to 5.2%), xylose (from 3.9 to 4.2%), and arabinose (2.0%) (Table 1). Galactose was previously revealed in the core oligosaccharides of the LPSs from *Salmonella enterica* and *E. coli* strains R1 and R2, whereas arabinose was found in the core oligosaccharide from *P. mirabilis* R45/1959 [17].

The most variable moiety of the LPS molecule is O-PS; modifications in its structure help the bacterium resist the immune system of the host plant and facilitate bacterial infection.

The O-PSs from *R. solanacearum* strains contained rhamnose as the predominant monosaccharide (86.8–92.6%), as well as glucose (2.1–9.0%) and xylose (0–11.1%), whereas arabinose was revealed only in the strain *R. solanacearum* 8115; glucosamine (8.05–19.7%) was an obligatory component of the O-PSs from all the strains studied (Table 1).

We elucidated the structure of the O-PSs from *R. solanacearum* strains using ^{1}H and ^{13}C NMR spectroscopy, Smith degradation analysis, methylation, and computer analysis [18]. The O-PSs consisted of repeating units of either linear tetrasaccharides (structures 1 and 2) or branched pentasaccharides (structure 3). Structures 1 and 2 differ both in their configuration (α or β) and in the type of bond (1 ⟶ 2 or 1 ⟶ 3) between glucosamine and rhamnose residues. Structure 3 is a xylose-containing form of structure 2. L-Xylose rarely occurs in bacterial polysaccharides and was earlier revealed only in the O-PS from *Stenotropnomonas* (formerly *Pseudomonas*) *maltophilia* NCTC 10257 [12].

R. solanacearum ICMP 6524 and 8115, structure 1 (100%) ⟶ 3)-α-D-GlcNAc-(1 ⟶ 2)-α-L-Rha-(1 ⟶ 2)-α-L-Rha-(1 ⟶ 3)-α-L-Rha-(1 ⟶

R. solanacearum ICMP 5712 and 8169, structure 2 (30%) ⟶ 3)-β-D-GlcNAc-(1 ⟶ 3)-α-L-Rha-(1 ⟶ 2)-α-L-Rha-(1 ⟶ 3)-α-L-Rha-(1 ⟶

and structure 3 (70%) ⟶ 3)-β-D-GlcNAc-(1 ⟶ 3)-α-L-Rha-(1 ⟶ 2)-α-L-Rha-(1 ⟶ 3)-α-L-Rha-(1⟶

4↑

|1

β-L-Xyl

R. solanacearum strains 5712 and 8169 differ in the type of the O-PS structure—linear (2) or branched and xylose-containing (3); therefore, these strains belong to different chemotypes.

Earlier, we revealed the occurrence of two or more types of O-PS structures in *P. syringae* [19]. However, it remains unknown whether such heterogeneity is determined by a concurrent presence of different O-PS chains or different repeating units in the chain. The biological role of this heterogeneity is unclear.

Thus, the studied biovar I strains of *R. solanacearum* are characterized by the following features of the LPS structure: (a) the strains are similar with respect to the predominant fatty acids of lipid A (3-OH-$C_{14:0}$, 2-OH-$C_{16:0}$, $C_{14:0}$, and $C_{16:0}$); (b) the major monosaccharides of the core oligosaccharides from all the strains studied are rhamnose, glucose, glucosamine, KDO, and heptose, although individual strains differ in the content of galactose, ribose, xylose, and arabinose; (c) the structure of the O-PS varies with the strain, and different types of the O-PS structure may occur in one strain.

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