# INVESTIGATION OF THE CORE OF THE LIPOPOLYSACCHARIDES OF *Yersinia pseudotuberculosis*

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Lipopolysaccharides have been isolated from two R mutants of the pseudotuberculosis microbe *Yersinia tuberculosis*, serovar VA. Mild acid hydrolysis followed by gel filtration on Sephadex G-25 gave the core oligosaccharides OS I and OS II. Complete acid hydrolysis showed that OS I consisted of residues of D-galactose, G-glucose, two heptoses (D-glycero-D-mannoheptose and L-glycero-D-mannoheptose), and D-glucosamine in a ratio of 1:2.5:4:1, while OS II consisted of residues of D-glucose, heptoses, and D-glucosamine in a ratio of 1:2.5:0.2. On the basis of the results of monosaccharide analysis, methylation, Smith degradation, etc., a partial structure of the core oligosaccharide of the LPS of *Y. pseudotuberculosis* has been put forward.

The core oligosaccharide, which is a component part of the lipopolysaccharides (LPS) of Gram-negative bacteria, together with the lipid of the A- and O-specific polysaccharides, is not distinguished by such a diversity of structures as is characteristic for the latter [1]. There is no information on the structure of the core LPS of the pseudotuberculosis microbe *Yersinia pseudotubeculosis* in the literature.

We have investigated two R mutants of *Y. pseudotuberculosis* obtained from serovar A (strain 2456).

The LPSs were isolated from a dry acetone powder of the microbial mass by Galanos's method [2].

The LPS I and LPS II (from the first and second mutants) were obtained with yields of 0.5% and 0.7%, respectively. LPS is contained residues of D-galactose, D-glucose, 2-acetamido-2-deoxy-D-glucose, and 2-keto-3-deoxyoctonic acid (KDO) and, in addition, residues of D-glycero-D-mannoheptose and L-glycero-D-mannoheptose in a ratio of ~1:3. LPS II had the same monosaccharide composition with the exception of the D-galactose residues.

The mild acid hydrolysis of the LPS I and LPS II with a 1% solution of acetic acid followed by gel filtration of the carbohydrate component (after the elimination of the lipid A by centrifugation) on Sephadex G-25 gave the corresponding core oligosaccharides (OS I and OS II). Complete acid hydrolysis and identification of the monosaccharides with the aid of gas–liquid chromatography (GLC) showed that OS I consisted of residues of D-galactose, D-glucose, two heptoses, and D-glucosamine in a ratio of 1:2.5:4:1, respectively, and OS II of residues of D-glucose, heptoses, and D-glucosamine in a ratio of 1:2.5:0.2.

It can be seen from the results of monosaccharide analysis that the LPS of the first mutant contained the complete core attached to lipid A, i.e., it belonged to the group of $R_a$ mutants which the biosynthesis of the O-specific chain is disturbed, while in the second mutant, probably, the biosynthesis of the core itself was disturbed and therefore its LPS contained an incomplete core (galactose was absent and the amount of amino sugar was lower).

To establish the link between the monosaccharide residues of the LPSs and the oligosaccharides obtained from them, the core was methylated by Hakomori's method [3] followed by methanolysis (or hydrolysis). The mixture of partially methylated monosaccharides so obtained was analyzed with the aid of chromato-mass spectrometry (GLC-MS). 2,3,4,6-Tetra-O-methyl-D-glucose, a 2,3,4,6,7-penta-O-methylheptose, a 2,3,4,6-tetra-O-methylheptose, a 2,6,7-tri-O-methylheptose, a 2,4,6-tri-O-methylheptose, a 6,7-di-O-methylheptose, and the

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TABLE 1. Partially Methylated Monosaccharides from Hydrolysates of Methylated Core Oligosaccharides (OS I and OS II) and of the Fragments Obtained From Them

| Partially Methylated Monosaccharides | OS I | OS II | OS IIa | Bonds |
|---|---|---|---|---|
| 2, 3, 4, 6-$OMe_4$-*D*-Glc* | + | + | + | *D*-Glc (1→ |
| 2, 3, 4, 6-$OMe_4$-*D*-Gal | + | — | — | *D*-Gal (1→ |
| 2, 3, 4, 6, 7-$OMe_5$-*DD*-Hep † | + | ++ | — | *DD*-Hep (1→ |
| 2, 3, 4, 6, 7-$OMe_5$-*LD*-Hep ‡ | + | — | ++ | *LD*-Hep (1→ |
| 2, 3, 6-$OMe_3$-*D*-Glc | ++ | — | — | →4)-*D*-Glc (1→ |
| 2, 4, 6-$OMe_3$-Man | — | — | ++ | →3)-Man (1→ |
| 2, 3, 4, 6-$OMe_4$-*LD*-Hep | ++++ | +++ | Сл. | →7)-*LD*-Hep (1→ |
| 2, 4, 6, 7-$OMe_4$-*LD*-Hep | + | + | +++ | →3)-*LD*-Hep (1→ |
| 2, 6-$OMe_2$-Man | — | — | ++ | →3)-Man (1→ |
| 4, 6-$OMe_2$-Hex | + | — | — | |
| 2, 6, 7-$OMe_3$-*LD*-Hep | +++ | +++ | Сл. | →3)-*LD*-Hep (1→ 4 |
| 2, 4, 6-$OMe_3$-*LD*-Hep | ++ | ++ | — | →3)-*LD*-Hep (1→ 7 4 |
| 6, 7-$OMe_2$-*LD*-Hep | + | + | — | →3)-*LD*-Hep (1→ 2 |
| 3, 4, 6-$OMe_3$-GlcN | + | + | — | GlcN (1→ |

*2,3,4,6-$OMe_4$-D-GLC – 2,3,4,6-tetra-O-methyl-D-glucose.
†2,3,4,6,7-$OMe_5$-DD-Hep – 2,3,4,6,7-penta-O-methyl-D-glycero-D-mannoheptose.
‡2,3,4,6,7,$OMe_5$-LD-Hep – 2,3,4,6,7-penta-O-methyl-L-glycero-D-mannoheptose, and so on.

Note. OS I) oligosaccharide core from the first mutant; OS II) oligosaccharide core from the second mutant; OS IIa) fragment obtained after the Smith degradation of OS II.

methyl ester of 4,5,7,8-tetra-O-methyl-KDO were detected in the LPSs of both mutants; and in LPS I, 2,3,4,6-tetra-O-methyl-D-galactose and 2,3,6-tri-O-methyl-D-glucose were detected in addition.

The oligosaccharide core can be separated into hexose and heptose moieties. In the oligosaccharide core of the first mutant, which had the complete core, the hexose moiety consisted of a D-galactose residue, a D-glucosamine residue, and three D-glucose residues, and the heptose moiety of four D-glycero-D-mannoheptose and L-glycero-D-mannoheptose residues.

It is obvious from the results of methylation (Table 1) that all the monosaccharide residues were present in the carbohydrate chain in the pyranose form. In the hexose moiety of the core, the D-galactose and D-glucosamine residues and also one D-glucose residue were present at nonreducing ends of carbohydrate chains.

In the heptose region, one of the heptose residues was present at a nonreducing end, in OS I this terminal heptose being represented only by D-glycero-D-mannoheptose (since only 2,3,4,6,7-penta-O-methyl-DD-heptose was identified, just as in the LPSs of all the serovars of Y. pseudotuberculosis present in the S form that were investigated), and in OS II both heptoses were present in the side chain (both 2,3,4,6,7-$OMe_5$-DD-Hep and 2,3,4, 6,7-$OMe_5$-LD-Hep were detected in the methanolysis products). In addition to this, there were monosubstituted heptose residues – in position 7 (2,3,4,6-$OMe_4$-Hep) and in position 3 (2,4,6,7-$OMe_4$-Hep); disubstituted pentose residues – in positions 3 and 4 (2,6,7-$OMe_3$-Hep) and in positions 3 and 7 (2,4,6-$OMe_3$-Hep); and also trisubstituted heptose residues – in positions 2, 3, and 4 (6, 7-$OMe_2$-Hep). Such a multiplicity of heptose derivatives showed the branched nature of the heptose moiety, the heptose residues of the main chain being partially substituted by a side-chain residue and by phosphate (OS I contained 2.46% of phosphorus).

The oligosaccharide of the core of the second mutant (OS II) was subjected to Smith degradation [4], which yielded a polyalcohol (OS IIa). In a hydrolysate of OS IIa, mannose, L-glycero-D-mannoheptose, glycerol, erythritol, and threitol were identified. Complete oxidation of the amino sugar was observed, and the glucose disappeared almost completely.

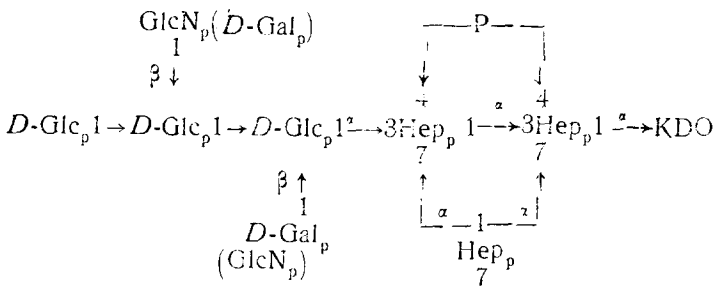
The ratio of the monosaccharides in the initial oligosaccharide (with xylose as internal standard) was: xylose—glucose—D-glycero-D-mannoheptose—L-glycerol-D-mannoheptose—glucosamine 1:1:0.8:2.1:0.2, and after degradation it was glycerol—erythritol—threitol—xylose—mannose—L-glycero-D-mannoheptose 1.5:0.4:0.3:1:1.3:1. The formation of threitol is explained by the presence in the initial polysaccharide of a L-glycero-D-mannoheptose residue substituted in position 7, and the formation of erythritol by the presence of a 7-substituted D-glycero-D-mannoheptose (or of 4-bound glucose). The appearance of mannose was due to the partial oxidation of a heptose substituent in position 3.

OS IIa was subjected successively to exhaustive methylation, methanolysis, and identification of the partially methylated monosaccharides with the aid of GLC-MS (Table 1). The mannose residues substituted in position 3 (2,4,6-$OMe_3$-Man) and in positions 3 and 4 (2,6-$OMe_2$-Man) were formed from the correspondingly substituted heptose residues of the main chain. The practically complete disappearance of 2,4,6-$OMe_3$-Hep fro the OS IIa methanolysate and the increase in the amount of 2,4,6,7-$OMe_4$-Hep indicated that the side chain in the heptose moiety was attached to position 7 of the heptose residues of the main chain. The results of Smith degradation confirmed that the structure of the core contained two nonoverlapping regions: hexose and heptose regions.

In order to establish the position of the phosphate residues, the core oligosaccharide (OS I) was subjected to dephosphorylation [5]. The amount of phosphorus fell from 2.46 to 1.04%. The oligosaccharide so obtained (OS Ia) was investigated by the methylation method. It was found that in OS Ia the amount of 2,4,6,7-$OMe_4$-Hep had increased, which indicates position 4 for the phosphate residue.

In order to determine the configurations of the glycosidic bonds we used a method based on the oxidation of the acetylated glycosides with chromium trioxide [6]. Under these conditions, β-glycosides undergo oxidation while α-glycosides are stable. The acetate of OS I was subjected to oxidation with chromium trioxide and the corresponding oxidized oligosaccharide (OS Ib) was obtained. Analysis of he monosaccharide composition of OS Ib by GLC showed the almost complete disappearance of the galactose and glucosamine residues, which indicates a possible β configuration of these residues and, probably, α bonds of the glucose and heptose residues (the amount and ratio of the monosaccharides in the initial OS I are given above; after oxidation the ratio of the remaining glucose and heptose residues was 1:2). In the methanolysis mixture of the completely methylated OS Ib, 2,3,4,6-$OMe_4$-D-Glc, 2,3,4,6,7-$OMe_5$-Hep, 2,3,6-$OMe_3$-D-Glc, 2,3,4,6-$OMe_4$-Hep, 2,6,7-$OMe_3$-Hep, and 2,4,6-$OMe_3$-Hep were identified. These results indicate that all the glycosidic bonds of the heptoses had the α configuration.

On the basis of the results obtained (from monosaccharide analysis, methylation, Smith degradation, etc.) the following structure may be proposed for the core oligosaccharide of the LPS of Yersinia pseudotuberculosis (serovar V):



On analyzing the results of the methylation of the LPSs of serovars Ib and III of the pseudotuberculosis microbe obtained previously [7, 8], it may be assumed that they all had cores of similar structure or, in any case, identical heptose regions, since in the methanolysis mixture obtained from the permethylated LPSs the same heptose derivatives were identified.

## EXPERIMENTAL

Mutants $R_1$ and $R_2$ were obtained by the repeated seeding of dissociated strain No. 2456 of serovar VA obtained from the International Yersinia Center (Paris, Prof. H. H. Mollaret).

The microorganism were grown at 37°C on a synthetic medium containing peptone, glucose, and inorganic salts [9]. The LPSs were isolated by extraction with a mixture of 90% phenol, chloroform, and petroleum ether (2:5:8 by volume) [2], followed by ultracentrifugation at 105,000 x g.

To determine the monosaccharide compositions, the LPSs (10 mg) and the polysaccharides (5 mg) were hydrolyzed with 0.5 N trifluoroacetic acid (TFA, 1 ml) in a sealed tube at 100°C for 3.5 h. The acid was eliminated by three to five evaporations with methanol in vacuum at 40-45°C. The monosaccharides were analyzed by GLC and by GLC-MS in the form of the corresponding polyol acetates.

Gel chromatography was performed on a column of Sephadexes G-25 and G-15 (2 × 65 cm) in pyridine-acetate buffer (10 ml of pyridine and 4 ml of acetic acid in 1 liter of water ), and GLC on a Pye-Unicam model 104 instrument with a flame-ionization detector using glass columns (4 mm × 150 cm) containing 3% of QF-1 on Gas Chrom Q, 100-120 mesh, (column A) and with 3% of OV-225 on Gas Chrom Q, 100-120 mesh, (column B). The acetates of the polyols from the monosaccharides were analyzed on columns A and B in the interval from 175 to 225°C (5°C/min). GLC-MS was performed on a LKB-9000S instrument using column A.

Isolation of the Core Oligosaccharides. The LPS I (500 mg) and LPS II (380 mg) were hydrolyzed with 1% acetic acid (50 and 40 ml) in the boiling water bath for 90 min. The lipid A that precipitated was separated off by centrifugation (5000 rpm) and was washed twice with water and was lyophilized (yields of lipid A 50 and 42%, respectively). All the supernatants were concentrated to small volume and were fractionated in a column of Sephadex G-25 in pyridine-acetate buffer. The first fractions were collected and lyophilized. As a result, the core oligosaccharides OS I and OS II were obtained (103 and 72 mg).

Methylation. The LPSs and the core oligosaccharides obtained from them (OS I, OS II, OS IIa, etc., 5-10 mg), after preliminary drying at 60°C over $P_2O_5$, were dissolved in dimethyl sulfoxide (0.5-1 ml) and were methylated with methyl iodide (0.5-1 ml) in the presence of the methylsulfinyl anion by the standard procedure [3]. After the end of methylation, the reaction mixture was diluted with a small amount of ice water and was extracted three times with chloroform. The chloroform and the dimethyl sulfoxide were eliminated by evaporation in vacuum. Half of the methylated compound was heated in a sealed tube with 0.5 N HCl in absolute methanol, the products were acetylated with acetic anhydride (0.3 ml) in pyridine (0.3 ml) at 100°C for 1 h, and the resulting acetates of methyl glycosides were analyzed by GLC-MS [7, 10] (column, 110-225°C, 5°C/min). The other half of the methylated product was subjected to formolysis (0.5 ml of 90% HCOOH, 100°C, 3 h), hydrolysis (0.5 ml of 0.25 N $H_2SO_4$, 12 h, 100°C), reduction with sodium tetrahydroborate, and acetylation. The partially methylated polyol acetates were also investigated by GLC-MS [11, 12] (column A, in the 120-225°C interval, 5°C/min).

Smith Degradation. OS II (100 mg) was oxidized with a 0.1 M solution of sodium metaperiodate in water (10 ml) at 20°C in the dark for 48 h, and then sodium tetrahydroborate (300 mg) was added to the solution in portions. After 22 h, the excess of $NaBH_4$ was decomposed with acetic acid and the whole mixture was passed through a column of Sephadex G-10 (2.4 × 64 cm) to free it from inorganic impurities and was lyophilized. This gave purified OS II, which was hydrolyzed with 0.5 M HCl (10 ml) at 20°C for 5 days. The modified core oligosaccharide (OC IIa) was isolated by chromatography on Sephadex G-15 (2 × 65 cm) in pyridine-acetate buffer and was lyophilized (yield about 40 mg).

Dephosphorylation. OS I (20 mg) was kept in a 45% aqueous solution of hydrogen fluoride (5 ml) in a polyethylene vessel at +4°C for 5 days [5]. Then the contents of the vessel were poured into ice water and were lyophilized. The residue was again dissolved in water, and a 2 M solution of LiOH was added (to pH 7), the precipitate was separated off by centrifugation, and the supernatant was evaporated to small volume and was subjected to gel filtration on Sephadex G-25, followed by lyophilization. This gave the phosphorylated OS I (OS Ia) with a yield of 16.5 mg.

Determination of the Configurations of the Glycosidic Bonds. OS I (3 mg) was dissolved in freshly distilled formamide (1 ml), and then pyridine (0.5 ml) and acetic anhydride (0.5 ml) were added and the mixture was left at room temperature for 2 days. It was poured into ice water, dialyzed, and lyophilized. The resulting acetate was dissolved in glacial acetic acid (0.3 ml), and 20 mg of chromium trioxide was added. The mixture was kept at 50°C for 2 h and was then diluted with water and extracted with chloroform. The extract was washed

with water and was evaporated to dryness. This gave the oxidized acetate of OS I (OS Ib). Part of the OS Ib was hydrolyzed with 0.5 N TFA, and another part was analyzed by the methylation method.

## SUMMARY

Lipopolysaccharides have been isolated from two R mutants of Yersinia pseudotuberculosis. The core oligosaccharides have been obtained by mild acid hydrolysis and their qualitative and quantitative monosaccharide compositions have been determined. The nature of the bonds between the monosaccharide residues has been determined by methylation and Smith degradation using chromato-mass spectrometry, and a partial structure of the core oligosaccharide of the lipopolysaccharides of the pseudotuberculosis microbe has been suggested.

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