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# STUDIES OF LIPOPOLYSACCHARIDES FROM *Yersinia pseudotuberculosis* SUBTYPES VA AND VB

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## ABSTRACT

Lipopolysaccharides (LPS) from various strains of *Yersinia pseudotuberculosis* type V have been isolated and characterised. Differences in sugar composition and serological activity of LPS from various strains within the same subtype of *Y. pseudotuberculosis* have been revealed.

## INTRODUCTION

Tsubakura *et al.*[1] divided *Y. pseudotuberculosis* type V into two subtypes (VA and VB). Samuelsson *et al.*[2] identified residues of ascarylose, L-fucose, D-mannose, D-glucose, D- and L-*glycero*-D-*manno*-heptose, and 2-acetamido-2-deoxy-D-glucose and D-galactose as constituents of lipopolysaccharides (LPS) of *Y. pseudotuberculosis* subtype VA.

LPS from *Y. pseudotuberculosis* VB did not contain ascarylose residues; in addition, D-galactose residues were found as a distinctive feature in comparison with subtype VA. The structural pattern of the trisaccharide repeating-unit of O-specific side-chains of the LPS from *Y. pseudotuberculosis* subtypes VA and VB was elucidated[2] by methylation studies of the LPS and the haptenic fraction. We now present comparative chemical and immunochemical studies of LPS from various strains of *Y. pseudotuberculosis* type V.

## RESULTS AND DISCUSSION

Comparative studies of LPS from various strains of *Y. pseudotuberculosis* subtypes VA and VB, obtained from France and Japan, have been carried out. The LPS were isolated by phenol–water procedure, usually in yields from 1.2 to 2.0% (Table I); the highest LPS content (3.4%) was found in strain No. 2454 of *Y. pseudotuberculosis* subtype VB.

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TABLE I

ANALYTICAL DATA FOR LPS FROM *Y. pseudotuberculosis* SUBTYPES VA AND VB

| *Strain* | *Yield (%)* | *Content (%)* | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *Sugars* | *Lipid A* | *Nucleic acids* | *KDO* | *3,6-Dideoxyhexoses* | *N-acetyl groups* | *O-acetyl groups* | *Organic P* |
| VA ≠ 12 | 1.4 | 48.7 | 32.0 | 1.0 | 4.8 | —[b] | 1.27 | — | 1.90 |
| VA ≠ 12[a] | 1.0 | 45.2 | 33.0 | 1.0 | 4.9 | — | 1.07 | — | 1.80 |
| VA No. 52 | 1.8 | 42.0 | 36.6 | 1.3 | 8.5 | 3.0 | 1.17 | — | 1.56 |
| VA No. 2456 | 2.0 | 37.3 | 41.1 | 1.1 | 8.9 | — | 0.78 | — | 1.50 |
| VA No. 2457 | 1.2 | 38.6 | 29.5 | 1.4 | 5.8 | 8.7 | 1.20 | — | 1.90 |
| VB-R2 | 1.9 | 36.2 | 35.0 | 1.5 | 5.9 | — | 1.27 | — | 1.40 |
| VB-R2[a] | 1.8 | 41.7 | 30.0 | 1.0 | 8.7 | — | 1.04 | — | 1.50 |
| VB No. 2454 | 3.4 | 57.4 | 34.0 | 0.7 | 5.7 | 10.9 | 0.75 | 2.75 | 2.10 |

[a]Strains passaged through mice. [b]—, Not detected.

TABLE II

AMINO ACID AND AMINO SUGAR CONTENTS (%) OF LPS OF SUBTYPES VA AND VB

| *Amino acid* | *VA ≠12* | *VA ≠12[a]* | *VA No. 52* | *VA No. 2456* | *VA No. 2457* | *VB-R2* | *VB-R2[a]* | *VB No. 2454* |
|---|---|---|---|---|---|---|---|---|
| Alanine | 0.03 | 0.12 | 0.12 | 0.10 | 0.02 | 0.02 | 0.68 | 0.03 |
| Glycine | 0.40 | 0.14 | 0.73 | 0.12 | 0.35 | 0.24 | 0.24 | 0.68 |
| Aspartic acid | 0.19 | — | 0.39 | 0.14 | 0.37 | 0.40 | 0.28 | 1.07 |
| Arginine | 0.22 | — | — | 0.50 | 0.35 | 0.35 | — | — |
| Lysine | 0.23 | 0.23 | 1.02 | 0.26 | 0.37 | 0.25 | 0.29 | 1.14 |
| Cysteic acid | 0.59 | — | — | — | 0.57 | 0.42 | — | — |
| Histidine | 0.07 | 0.10 | 0.04 | 0.21 | 0.07 | 0.06 | 0.20 | — |
| Leucine | 0.18 | — | 0.04 | 0.12 | 0.30 | 0.17 | — | — |
| Isoleucine | 0.12 | — | 0.05 | 0.04 | 0.18 | 0.14 | — | — |
| Valine | 0.13 | — | — | 0.06 | 0.16 | 0.11 | — | — |
| Glutamic acid | 0.14 | 0.07 | 0.10 | 0.10 | 0.07 | 0.28 | — | — |
| Serine | 0.30 | 0.08 | 0.16 | 0.08 | 0.09 | 0.17 | 0.19 | — |
| Threonine | 0.16 | 0.03 | 0.06 | 0.18 | 0.18 | 0.15 | 0.07 | — |
| Cysteine | 0.20 | — | — | 0.29 | 0.06 | 0.04 | — | 0.02 |
| GlcN | 3.00 | 2.96 | 3.40 | 3.00 | 1.50 | 2.00 | 1.9 | 4.64 |
| GalN | 0.40 | 0.7 | 1.02 | 0.40 | 1.70 | 1.80 | 1.9 | 0.60 |

[a]Strains passaged through white mice.

The protein portions of the various LPS contained the same amino acids (Table II). Quantification indicated a lower content of amino acids in the protein portions of LPS isolated from strains passaged through mice. It should be noted that the protein part of the LPS isolated from strain No. 2454 of subtype VB contained only alanine, glycine, aspartic acid, lysine, and cysteine.

TABLE III

SUGAR COMPOSITION OF *Y. pseudotuberculosis* SUBTYPES VA AND VB

| *Strain* | *Molar %*[a] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *Asc* | *Tyv* | *Rha* | *Fuc* | *Man* | *Glc* | *Gal* | *Hep* D-D | *Hep* L-D |
| VA ≈ 12 | — | — | 13.6 | 6.8 | 5.6 | 5.4 | 4.8 | 3.0 | 9.5 |
| VA ≈12[b] | — | — | 9.8 | 5.5 | 6.2 | 7.7 | 6.2 | 2.4 | 7.3 |
| VA No. 2456 | — | — | — | — | 0.4 | 13.0 | 5.7 | 5.9 | 12.2 |
| VA No. 2457 | 3.4 | — | — | 14.3 | 7.3 | 3.7 | — | 2.4 | 7.5 |
| VA No. 52 | 2.3 | — | — | 7.2 | 3.3 | 5.9 | 3.9 | 4.1 | 15.2 |
| VB-R2 | — | — | — | 10.5[c] | 5.4 | 5.0 | 2.6 | 2.9 | 8.8 |
| VB-R2[b] | — | — | — | 11.7[c] | 3.16 | 7.3 | 5.2 | 3.1 | 8.4 |
| VB No. 2454 | 8.3 | 9.8 | 13.2 | — | 11.7 | 9.2 | 13.2 | — | 1.9 |

[a]Calculated from g.l.c. data (as alditol and aldononitrile acetates) and from the total content of monosaccharides determined by the phenol–sulphuric acid procedure. [b]Strains passaged through white mice. [c]Mixture of fucose and unidentified sugar.

The total contents of amino sugars ranged from 3.2 to 5.2%. Most of the LPS isolated were shown to comprise residues of 2-amino-2-deoxyglucose (glucosamine) and traces of 2-amino-2-deoxygalactose (galactosamine). However, LPS from strains VA No. 2457, VA ≈12, and VB-R2 contained residues of glucosamine and galactosamine in the ratio 1:1. The content of *N*-acetyl groups was 1.0–1.3%.

The relative molar ratios of sugars for the various LPS, identified by paper and gas–liquid chromatography, are given in Table III. Glucose, galactose, D- and L-*glycero*-D-*manno*-heptose, glucosamine, and ketodeoxyoctonic acid (KDO) are known to be core monosaccharides. They were present in each LPS, except LPS VB No. 2454 which did not contain D-*glycero*-D-*manno*-heptose residues.

The LPS from strain VB No. 2456 (R-form) contained only the core sugars. Fucose, mannose, ascarylose, and galactosamine were identified in LPS VA No. 2457 and VA No. 52 in addition to the core sugars. The occurrence of ascarylose was confirmed by its isolation, followed by determination of optical rotation and identification by g.l.c.–m.s. of its methyl glycoside acetate (major fragments at *m/e* 43, 74, 100, 103, 116, 126, 143, 186, and 215).

In addition to the core sugars, rhamnose, fucose, and mannose were found in the LPS of *Y. pseudotuberculosis* subtype VA ≈12; rhamnose, mannose, galactose, and tyvelose were detected in the LPS of subtype VB strain No. 2454. Fucose, mannose, galactosamine, and an unidentified sugar were detected in the LPS of subtype VB-R2. The retention times (g.l.c.) of the unidentified sugar ($R_{Rha}$ 1.1, p.c.) as its alditol and aldononitrile acetate were the same as those for fucose. Probably this sugar is a 6-deoxyhexose.

Strain No. 2455 of *Y. pseudotuberculosis* subtype VB failed to grow in agar. This micro-organism was not subjected to further investigation.

The LPS isolated from various strains of *Y. pseudotuberculosis* differed in sugar composition. The LPS of VA No. 2457 and No. 52 (France) contained ascarylose, fucose, mannose, glucose, galactose, 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-galactose, and KDO. The LPS of subtype VA (Japan) differed in sugar composition, containing L-rhamnose instead of ascarylose. An unidentified sugar was detected in the LPS of strain VB-R2, while the LPS of VB No. 2454 lacked D-*glycero*-D-*manno*-heptose, characteristic of all LPS of *Y. pseudotuberculosis*, and contained tyvelose. This LPS also contained *O*-acetyl groups (2.57%), thus indicating the presence of acetylated sugar residue in the carbohydrate moiety.

The O-specific side-chains of LPS VB No. 2454 consisted of tyvelose, rhamnose, mannose, and galactose residues in molar ratios of 1:1:1:1 and were isolated by molecular-sieve chromatography on Sephadex G-50.

Culture-morphological, serological, and biochemical properties of various strains of *Y. pseudotuberculosis* type V were studied. All of the original strains gave dry, flattened colonies with a granular surface, dense centre, and light peripheral zones, having uneven extremities. The S-form colonies gave a copper-green sparkle under a slanting light ray[3]; colonies of R- and transient-forms gave a brownish-yellow sparkle (strains 2456 and 2457). Micro-organisms of strain VB No. 2454 gave a sparkle similar to that of the S-form, but its shade was not characteristic of *Y. pseudotuberculosis*.

Homogeneous growth was observed in beef-extract broth, except for strain VA No. 2456, which gave a sediment. The colours of all strains were characteristic of *Y. pseudotuberculosis*, except for strain VB No. 2454 which differed in its culture-morphological and biochemical properties from other strains.

Titres in the passive hemagglutination reaction of antisera obtained by immu-

TABLE IV

ANTIGENIC RELATIONSHIP BETWEEN STRAINS OF *Y. pseudotuberculosis* TYPE V IN PASSIVE HEMAGGLUTINATION (IHAR) AND PRECIPITATION[a] (PR) REACTIONS

| *LPS* | *Sera* *VA ≠12* | | *VB-R2* | | *VA No. 2456* | | *VA No. 2457* | | *VA No. 52* | | Y. pestis | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *IHAR* | *PR* | *IHAR* | *PR* | *IHAR* | *PR* | *IHAR* | *PR* | *IHAR* | *PR* | *IHAR* | *PR* |
| VA ≠ 12 | 1:320 | + | 1:80 | — | [b] | — | [b] | — | 1:800 (traces) | — | 1:160 | — |
| VB-R2 | 1:1280 | — | 1:1280 | — | [b] | — | [b] | — | 1:80 | — | 1:160 | — |
| VA No. 2456 | 1:320 | — | [b] | | 1:10240 | — | [b] | — | 1:1600 (traces) | — | 1:1280 | + |
| VA No. 2457 | 1:160 | — | 1:320 | — | 1:320 | + | 1:12800 | + | 1:12800 | + | 1:1280 | — |
| VA No. 52 | 1:640 | — | 1:80 | — | 1:40 | | 1:640 | + | 1:3200 | + | 1:1280 | — |
| VB No. 2454 | [b] | — | [b] | — | [b] | — | [b] | — | [b] | — | [b] | |

[a] + and — signs show positive and negative precipitation reactions, respectively. [b] Did not work.

nisation of rabbits by intravenous injection of strains of *Y. pseudotuberculosis* VA and VB serotypes are shown in Table IV.

All LPS, except that from VB No. 2454, possessed cross-reactivity with antiserum to *Yersinia pestis* in the passive hemagglutination test. Apparently this strain does not belong to the *Y. pseudotuberculosis* group.

The isolated LPS formed a single precipitation band with homologous antisera in the precipitation test. The majority of LPS gave one precipitation band during immunoelectrophoresis, except the LPS of VA No. 2456 which formed two uncharged precipitation bands.

Only the LPS of VA No. 2456 gave a precipitation band with plague serum. The presence of antigenic differences between type V LPS from Japan and France was found during the passive hemagglutination test.

The precipitation reaction for the LPS proved to be more specific, showing a positive test with homologous antisera only. There were no cross reactions between LPS of various subtypes.

## EXPERIMENTAL

*General conditions.* — Paper chromatography was performed on Whatman 3MM and Filtrak FN II papers by two-fold development with 1-butanol–pyridine–water (6:4:3). Neutral sugars were detected with alkaline silver nitrate and with aniline hydrogen phthalate, KDO with thiobarbituric acid, and amino sugars with ninhydrin in acetone. G.l.c. was performed on a Pye–Unicam 104 chromatograph fitted with a flame-ionisation detector and a column packed with 3% of QF-1 on Gas-Chrom Q (100–120 mesh); temperature programme, 175→225° at 5°/min. Combined g.l.c.–m.s. of monosaccharides as the alditol acetates, methyl glycosides, and aldononitrile acetates was performed on an LKB-9000 instrument, using 3% of QF-1 on Gas Chrom Q (100–120 mesh). Optical rotations were measured with an automatic Perkin–Elmer 141 polarimeter.

The total content of neutral sugars was determined by the phenol–sulphuric acid procedure[4], and 3,6-dideoxy sugars were detected by a modified TBA procedure[5]. Acyl groups were determined by the method of Trutnovsky *et al.*[6]. Lipid A content in each LPS was estimated by weighing the precipitate formed on hydrolysis of the LPS with 1% acetic acid at 95° for 3 h.

*Micro-organisms.* — The strains of *Y. pseudotuberculosis* subtype VA No. 52, No. 2456 (according to Thal, 232), and No. 2457 (Thal, 420), and strains of subtype VB No. 2455 (Thal, 460) were provided by Professor H. H. Mollaret (France). Strains VA ≈12 and VB-R2 were provided by M. Tsubakura (Japan). The strains were cultured on plain agar.

To obtain S-forms, the initial strains were treated with complement[7] or passaged through mice.

*Preparation of LPS.* — To obtain the LPS, the bacteria were grown in a fermenter on a nutrient medium as described earlier[8]. The LPS were isolated from dry

bacterial cells by the phenol–water procedure; nucleic acids were removed by triple ultracentrifugation at 105,000*g*. The yields of LPS are listed in Table I.

*Acid hydrolysis.* — Each LPS (10 mg) was hydrolysed with 0.5M $H_2SO_4$ (1 ml) for 4 h at 100°; the hydrolysate was neutralised with $BaCO_3$ and deionised with KU-2($H^+$) resin. Identification of sugars was performed by p.c. followed by g.l.c. of the alditol, aldononitrile, or methyl glycoside acetates.

Amino sugars in hydrolysates of the LPS (5 mg) were determined with 4M HCl (0.5 ml) at 100° for 4 h. Amino acids were identified in hydrolysates of LPS obtained by using 6M HCl for 6 and 12 h at 100°. Hydrochloric acid was removed by co-evaporation with methanol *in vacuo*. An amino acid analyser (LKB-Biocal 3201) was used for amino sugar and amino acid determination.

*Partial hydrolysis.* — LPS VB No. 2454 (200 mg) was hydrolysed with 0.125M $H_2SO_4$ (20 ml) for 30 min at 100°. The mixture obtained was centrifuged to yield lipid A (68 mg). The supernatant solution was neutralised as described above and concentrated to 2 ml, and then ethanol (10 ml) was added. The precipitate was removed, and the material (85 mg) in the ethanolic solution was subjected to preparative p.c., to give tyvelose (8.5 mg), $[\alpha]_{578}^{20}$ +19.5° (*c* 0.65, water), $R_{Rha}$ 1.3.

LPS VA No. 52 (200 mg) was hydrolysed as described above; the ethanol extract (100 mg) was isolated, and ascarylose (5 mg), $[\alpha]_{578}^{20}$ −18° (*c* 0.5, water), $R_{Rha}$ 1.3, was obtained by triple preparative p.c.

LPS VB No. 2454 (350 mg) was treated with 1% acetic acid (45 ml) at 95° for 3 h. The water-insoluble lipid A was separated by centrifugation; yield, 152.6 mg. The supernatant solution was freeze-dried to yield the haptenic fraction (176 mg). A solution of the hapten in water (1 ml) was subjected to gel filtration on a column (65 × 1.8 cm) of Sephadex G-50 with pyridine–acetic acid–water (10:4:986). Fractions were analysed by the phenol–sulphuric acid method. Yields of fractions I, II, and III were 74, 54.4, and 5 mg, respectively.

*Serological studies.* — Antisera to various strains of *Y. pseudotuberculosis* were prepared by the immunisation of rabbits by the method of Thal[9]. Commercial plague serum having an agglutination titre of 1:1280 was used.

The previously described procedures used were as follows: precipitation in agar and immunoelectrophoresis in veronal buffer (pH 8.4). LPS treated with 0.02M NaOH at 37° for 18 h were used in passive hemagglutination tests.

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