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Note

Sugar composition of lipopolysaccharide from *Haemophilus paraphrophilus*

ILIA BRONDZ*,*

Department of Chemistry, University of Oslo, Blindern, Oslo 3 (Norway)

and

INGAR OLSEN

Department of Microbiology, Dental Faculty, University of Oslo, Blindern, Oslo 3 (Norway)

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Haemophilus paraphrophilus is a short, regular, Gram-negative rod with occasional filamentous forms which is found as a member of the normal flora of the oral cavity and pharynx [1]. It may cause subacute endocarditis, brain abscess and paronychia, and has been isolated from osteomyelitis of the jaw, inflamed appendix, urine and vagina [2].

Genetically, *H. paraphrophilus* is closely related to *H. aphrophilus* [3], and both are very similar biochemically [2]. Sneath and Johnson [4], in their taxonomic studies on *Actinobacillus*, *Haemophilus* and *Pasteurella* strains, reported that *H. aphrophilus* and *H. paraphrophilus* strains formed a single cluster. Furthermore, Tanner et al. [5] found no clear separation of *H. aphrophilus* and *H. paraphrophilus* by cluster analysis of phenotypic features and DNA/DNA homology.

Recently, we have been able to differentiate between *H. aphrophilus* and *H. paraphrophilus* by means of whole-cell methanolysates derivatized with trifluoroacetic acid anhydride (TFAA) [6]. Whereas *H. paraphrophilus* contains both D-glycero- and L-glycero-D-mannoheptose, *H. aphrophilus* contains only L-glycero-D-mannoheptose. Usually, D-glycero-D-mannoheptose is regarded as a sugar constituent in the core of lipopolysaccharide (LPS) from Gram-negative bacteria [7], but other bacterial sources of this aldoheptose have also been

*Present address: Department of Occupation Medicine, Telemark Central Hospital, Porsgrunn, Norway.

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detected [8]. In the present study, whole, phenol-extracted LPS has been examined to see whether the sugar composition of this preparation may assist in the differentiation between *H. paraphrophilus* and *H. aphrophilus*, and whether LPS is the primary source of aldoheptoses in *H. paraphrophilus*. By examining the sugar composition of LPS in *H. paraphrophilus*, it was also hoped to get some idea of the structure of LPS in this organism.

EXPERIMENTAL

Bacterium

The type strain (ATCC 29241) of *H. paraphrophilus* was obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). After reconstitution from the lyophilized state, it was cultivated at 37°C in Brain Heart Infusion® (Difco, Detroit, MI, U.S.A.) broth supplemented with filter-sterilized NAD (1 mg/ml) and haemin (5 mg/ml) under 10% carbon dioxide in air for five days. The organism was maintained anaerobically (80% nitrogen, 10% hydrogen, 10% carbon dioxide) on chocolate—agar plates at 35°C and transferred weekly. Stock cultures were kept in liquid nitrogen.

Preparation of lipopolysaccharide

LPS from *H. paraphrophilus* was isolated by the phenol—water method as detailed previously [9].

Methanolysis

Samples (0.33 mg) of LPS were methanolysed (2 *M* hydrochloric acid in 2 ml of anhydrous methanol for 24 h at 85°C) [10]. After cooling, the methanolysate was concentrated, while kept on ice, by a stream of nitrogen. Chloroform (Fluka, Buchs, Switzerland), 2 ml, was added and the mixture transferred to a separating funnel, followed by two 1-ml batches of chloroform for washing the methanolysis tube. Water, 2 ml, was added twice. After separation of water and organic phases [9], the water phases were pooled and lyophilized.

Derivatization

The lyophilized water phase (50 μg) was transferred to a 100-μl screw-capped vial with septum (Hewlett-Packard Models). A PTFE liner was pierced with an 8-mm circular bur and placed under the rubber liner of the screw cap to prevent contamination with rubber during hydrolysis. TFAA (Fluka) in acetonitrile (Rathburn, U.K.), 1:3, was transferred to the microvial with a glass-tipped micropipette. The vial was filled completely with 100 μl of derivatization fluid. After closing the vial, the sample was derivatized for 3 min at 90°C. Electronic temperature monitoring was used to ascertain that these conditions were maintained inside the vial. Conditions of hydrolysis diverging from these tended to change the interrelationship between the derivatization products formed [10]. Immediately after derivatization, the microvial was cooled on ice. Synthetic sugars and reference LPSs were methanolysed as described above.

Temperature monitoring

The device constructed (E. Brondz, Department of Physics, University of

Oslo, Oslo, Norway) for continuous temperature monitoring consisted of a solid-state temperature sensor of type LM 135H (National Semiconductor Corporation, Santa Clara, CA, U.S.A.), a preamplifier based on a Precision Instrumentation amplifier of type AD524 (Analog Devices, Norwood, MA, U.S.A.), and a multimeter used as a display device. The temperature sensor enabled us to limit the calibration procedure to compare the standard mercury precision thermometer with the freezing point of water (0°C). This was achieved by trimming the resistor R_3 (Fig. 1a). The temperature was read in a reference vial (Fig. 1b) completely filled with derivatization fluid, care being taken to prevent the formation of bubbles. The precision was 0.1°C.

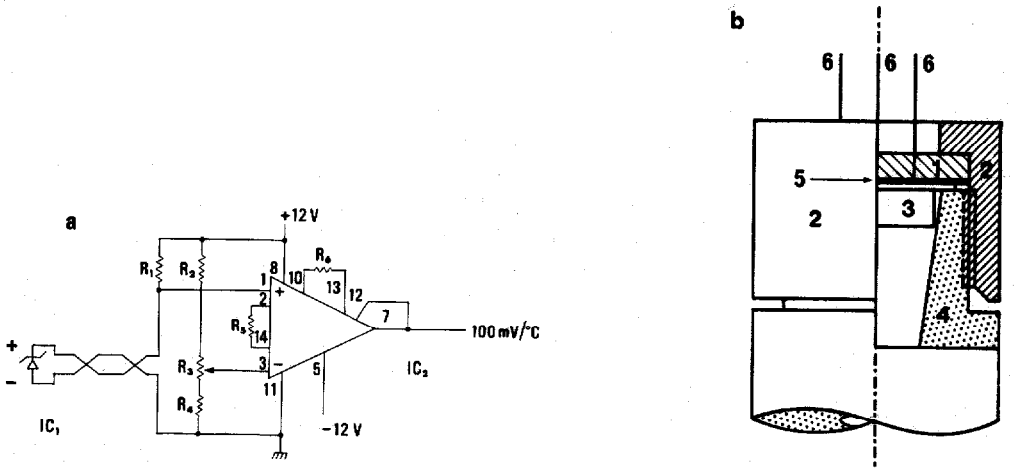

Fig. 1 (a). Wiring of the solid-state temperature sensor and preamplifier. The upper temperature boundary was 110°C, and the tolerance of the resistors was 2%. R_1 and R_2: 15 kΩ; R_3: 1 kΩ; R_4: 4.3 kΩ; R_5: 100Ω; R_6: 100 kΩ; IC_1: LM 135H precision temperature sensor; IC_2: AD 524JD instrumentation amplifier. (b) Connection between temperature sensor and microvial cap. 1 = Screw-capped vial septum; 2 = rubber screw-capped vial; 3 = solid-state thermal sensor; 4 = body of vial; 5 = PTFE liner; 6 = IC_1 terminals.

Reference compounds

Sigma (St. Louis, MO, U.S.A.) provided α-D(+)-fucose, D(+)-galactose, α-D(+)-glucose, D(+)-galactosamine, D(+)-glucosamine, D(+)-mannose and α-L-rhamnose. Natural galactose, glucosamine, L-glycero-D-mannoheptose, mannose and rhamnose were identified from LPS (Sigma) of *Escherichia coli* [11] and *Salmonella typhimurium* [12]. D-Glycero-D-mannoheptose was determined from *Chromobacterium violaceum* cells [13] and from *Yersinia pestis* LPS [14]. *Ch. violaceum* and N-glucosamine myristate were provided by O. Lüderitz and U. Meier (Max-Planck-Institut für Immunbiologie, Freiburg, F.R.G.) and *Y. pestis* LPS by B. Lindberg and P.-E. Jansson (Arrheniuslaboratoriet, University of Stockholm, Sweden).

Gas chromatography

A type 5040A Hewlett-Packard (Avondale, PA, U.S.A.) gas chromatograph with an electronic integrator was used. The Chrompack (Middelburg, The

TABLE I

PERCENTAGE SUGAR COMPOSITION OF PHENOL-EXTRACTED LIPOPOLYSACCHARIDE FROM *HAEMOPHILUS PARAPHROPHILUS*

Mean values are given (n = 18; S.D. ≤ 5%).

Sugar	Composition (%)
Rhamnose	Trace amount (< 0.1%)
Fucose	Trace amount (< 0.1%)
Galactose	15.2
Glucose	65.9
D-Glycero-D-mannoheptose	8.0
L-Glycero-D-mannoheptose	8.7
Galactosamine plus glucosamine	1.4

Netherlands) CP-Sil 5 (polydimethylsiloxane) glass-capillary column (30 m × 0.22 mm I.D.) used had a film thickness of 0.13 μm. Helium served as the carrier gas at a flow-rate of 2 ml/min. The pressure at the inlet of the column was 151.5 kPa. The temperature of the injector and the flame ionization detector was 200 and 220°C, respectively. Programme: hold 2 min at 90°C, then from 90°C to 190°C and 4°C/min with the attenuator set at 4. The chart-paper speed was 10 mm/min. The sample (0.2 μl) was delivered by splitless injection. The identity of the methanolysed and derivatized sugars was determined tentatively with gas chromatography. The full identity of the derivatives had previously been established with gas chromatography—mass spectrometry [10]. The batch of LPS to be examined was divided into three parts, each of which was methanolysed. Each methanolysate was divided into three parts, all of which were derivatized separately. From each derivative, two runs were made on the gas chromatograph. Accordingly, the figures presented in Table I are mean values of eighteen runs on the gas chromatograph. Quantitation of sugars and fatty acids was performed as described previously [6].

RESULTS AND DISCUSSION

The distribution of sugars in whole LPS from the type strain of *H. paraphrophilus* is shown in Table I. Glucose was present in the largest concentrations, followed by galactose and then D-glycero- and L-glycero-D-mannoheptose in similar amounts. Only trace amounts of rhamnose and fucose could be detected. A gas chromatogram of the TFAA-derivatized methanolysate of whole LPS is shown in Fig. 2.

The present study provided information on the sugar composition of whole LPS from *H. paraphrophilus*. To the best of our knowledge, the sugar composition of LPS from this organism has not previously been reported. A noteworthy feature of this study was the finding of both D-glycero- and L-glycero-D-mannoheptose in LPS from *H. paraphrophilus*, Previously, we found D-glycero- and L-glycero-D-mannoheptose in LPS from *A. actinomycetemcomitans*, and only L-glycero-D-mannoheptose in LPS from *H. aphrophilus* [10]. These organisms, as well as *H. paraphrophilus*, are members of the family *Pasteurellaceae*. Our findings suggested that D-glycero-D-mannoheptose of LPS can be

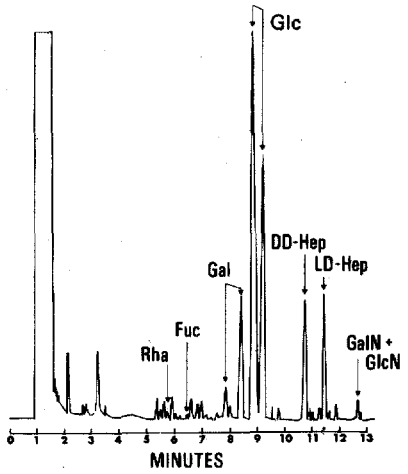

Fig. 2. Gas chromatogram showing the sugar composition of lipopolysaccharide from *Haemophilus paraphrophilus* (type strain). Peaks: Rha = rhamnose; Fuc = fucose; Gal = galactose; Glc = glucose; DD-Hep = D-glycero-D-mannoheptose; LD-Hep = L-glycero-D-mannoheptose; GalN = galactosamine; GlcN = glucosamine.

used to distinguish *H. paraphrophilus* from the closely related *H. aphrophilus*. A similar conclusion was drawn from a series of reference and laboratory strains of *H. paraphrophilus* and *H. aphrophilus*, the whole cells of which were analysed for their sugar composition [6]. The distinction between *H. paraphrophilus* and *H. aphrophilus* has been questioned [5]. Actually, it has been claimed that *H. paraphrophilus* would be erroneously speciated by nearly all clinical laboratories [15]. If the cellular sugar composition and perhaps also the structure of LPS is used as a basis for differentiation, *H. paraphrophilus* seems to be more closely related to *A. actinomycetemcomitans* than to *H. aphrophilus*. Nevertheless, it was also possible to distinguish *H. paraphrophilus* from *A. actinomycetemcomitans* owing to a higher content of rhamnose and fucose in LPS from *A. actinomycetemcomitans* than in LPS from *H. paraphrophilus*. Other important species of the family *Pasteurellaceae*, such as *H. influenzae* type b, *P. haemolytica*, *P. multocida* and *P. ureae*, have also been found to contain D-glycero-D-mannoheptose [6]. If D-glycero-D-mannoheptose can be regarded as a taxonomic marker in *Pasteurellaceae*, it would appear that *H. aphrophilus* occupies a specific niche in this family distant from other clinically important species.

The quantitative relationship between D-glycero- and L-glycero-D-mannoheptose in *H. paraphrophilus* was approximately the same in LPS as in whole cells [6]. This suggested that LPS is the primary source of D-glycero-D-mannoheptose in *H. paraphrophilus*. LPS is also the primary source of this aldoheptose in *A. actinomycetemcomitans* [16].

In the present study it was possible to make sugar analyses from as little as 50 μg of extracted and freeze-dried water phase LPS. It is important to reduce the biomass required for sugar analyses since the yield of LPS from facultatively anaerobic bacteria prepared with the phenol—water procedure can be relatively low.

CONCLUSIONS

(1) Phenol—water-extracted LPS from *H. paraphrophilus* contained glucose (65.9%), galactose (15.2%), L-glycero-D-mannoheptose (8.7%), D-glycero-D-mannoheptose (8.0%), galactosamine and glucosamine (1.4%), and trace amounts of rhamnose and fucose.

(2) D-Glycero-D-mannoheptose in *H. paraphrophilus* may be used to differentiate this organism from the closely related *H. aphrophilus* where it is absent.

(3) Lipopolysaccharide seems to be the major source of D-glycero-D-mannoheptose in *H. paraphrophilus.*

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