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Structural and serological characterization of *Hafnia alvei* lipopolysaccharide core region

Czesław Ługowski[a], Tomasz Niedziela[a], Wojciech Jachymek[a], Agnieszka Klonowska[a], Anna Czarny[a], Stefan Rowiński[a], Carl Petersson[b] and Lennart Kenne[b]

[a]*Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Czerska 12, 53–114 Wrocław, Poland*

[b]*Swedish University of Agricultural Sciences, P.O. Box 7015, S-750 07 Uppsala, Sweden*

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The structures and serological activities of core oligosaccharide of *Hafnia alvei* strains have been investigated. Methylation analysis, NMR spectroscopy and various specific degradation procedures were the principal methods used. It is concluded that, core hexasaccharides are identical in the lipopolysaccharides tested and are built of two glucose, three heptose and one 2-keto-3-deoxyoctulosonic acid residues. The antiserum raised against the ATCC 13337 oligosaccharide core-tetanus toxoid conjugate cross-reacted strongly with all lipopolysaccharides used as antigens in ELISA test, suggesting that this core region is the common structure in the *Hafnia* genus.

Hafnia alvei is a typical member of *Enterobacteriaceae*. Bacteria of this species are Gram-negative, motile, peritrichously flagellated, rarely encapsulated rods. *H. alvei* is an opportunistic pathogen found in some incidences of nosocomial infections; cases of septicemia caused by these bacteria have also been reported [1]. The detailed structure of the core region has not been determined yet for any *H. alvei* lipopolysaccharide (LPS)[1] except for some data concerning a core hexasaccharide in strain 2 and a core trisaccharide in strains 32 and 1192 [2] and strains 2 and 1211 [3]. Since the core region plays an important role in bacterial physiology and interaction with the host it was of current interest to characterise this part of the endotoxin of six *H. alvei* strains: 32, 744, 1200, 1203, 1209 and 1213.

In our earlier studies on the monospecific antiserum raised against *Shigella sonnei* phase II core oligosaccharide-protein conjugate a distinct relationship between serological reactions and the structure of core regions of lipopolysaccharides was observed [4]. In the present work we used the monospecific anti-*Hafnia alvei* ATCC 13337 core-tetanus toxoid serum to examine the relationship between different *H. alvei* strains.

EXPERIMENTAL

H. alvei strains 32 and 744 were obtained from the collection of the Institute of Immunology and Experimental Therapy (Wrocław) and strains: 1200, 1203, 1209, 1213 and ATCC 13337

[1]Abbreviations: COSY, two-dimensional NMR correlation spectroscopy; GLC-MS, gas liquid chromatography-mass spectrometry; HMQC, heteronuclear multiple quantum correlation; Kdo, 2-keto-3-deoxyoctulosonic acid; LPS, lipopolysaccharide; NOESY, nuclear Overhauser enhancement spectroscopy; TOCSY, totally correlated spectroscopy.

were from the Pasteur Institute (Paris). The bacteria were grown at 37°C in the synthetic minimal medium containing casein peptone (0.5%) and nicotinic acid (0.00001%). Cultures were aerated by shaking. After 24 h of growth the bacteria were harvested by centrifugation, washed three times with saline, three times with acetone and dried.

Lipopolysaccharide (LPS) was extracted from bacterial cells by the hot phenol/water method [5], purified by repeated ultracentrifugation (100000 × *g*, 5 h) and freeze-dried.

NMR spectroscopy. NMR spectra were obtained for ^2H_2O solutions at 35°C on Varian Unity 500 and 600 and Varian VXR-400 spectrometers using sodium 3-trimethylsilylpropionate-d_4 (δ_H 0.00) and acetone (δ_C 31.00) as internal references and 85% phosphoric acid (δ_P 0.00) as external reference. The signals were assigned by one- and two-dimensional procedures (dq-COSY, relayed COSY, double relayed COSY, TOCSY, NOESY and HMQC).

Sugar and methylation analyses. A solution of the sample (about 0.5 mg) in 2 M aqueous CF_3CO_2H (1.0 mL) was kept in a closed vial at 120°C for 2 h. The sugars in the hydrolysate were then converted to alditol acetates by conventional methods. The absolute configurations of the sugars were determined as described by Gerwig *et al.* [6, 7] using (+)-2-butanol for glycosylation.

Methylations were performed according to Hakomori [8]. Products were recovered by reversed phase chromatography on Sep-Pak C_{18} cartridges. The permethylated material was hydrolysed with 2 M aqueous CF_3CO_2H at 120°C for 2 h. The partially methylated sugars in the hydrolysate were then converted to alditol acetates by conventional methods. Reduction of ester groups with "Superdeuteride" ($LiB(C_2H_5)_3D$) after methylation of polysaccharide was carried out according to Bhat *et al.* [9].

Dephosphorylation of core OS. Crude core OS (Fraction III from the fractionation on Bio-Gel P-10) (30 mg) was incubated with 48% hydrofluoric acid (3 mL) at 4°C for 3 days with stirring. The acid was evaporated in a stream of nitrogen at room temperature and the residue dissolved in water (5 mL) and freeze-dried. The product was purified by column chromatography on Bio-Gel P-10 and freeze-dried.

Complete reduction (keto and carboxyl groups) of the Kdo residue in core OS was performed as described earlier [2].

Smith degradation of the core oligosaccharide was performed essentially as described by Kenne *et al.* [10].

Localization of the phosphate groups in core OS. Core OS (2 mg) was methylated and purified as described above and then incubated with 48% hydrofluoric acid (1 mL). The product was alkylated using ethyl iodide as described [8], hydrolysed and the sugars converted to alditol acetates and analysed by GLC-MS.

The conjugate of Hafnia alvei ATCC 13337 OS core with tetanus toxoid was prepared by reductive amination according to Ługowski *et al.* [4].

Immunization procedure. Rabbits were immunized by injection into footpads of 50 μg of the conjugate suspended in Freund complete adjuvant on days 0 and 21. The animals were bled 14 days after the second injection.

Immunoblotting was carried out as previously described [4].

Enzyme linked immunosorbent assay (ELISA) was performed by the modified method of Voller *et al.* [11] as previously described [12].

RESULTS AND DISCUSSION

Enzymatic and chemical analysis of core oligosaccharides gave D-glucose, L-*glycero*-D-*manno*-heptose (LDHep), Kdo, phosphate and ethanolamine in the molar ratio of 2:1:1:2:1. Methylation analysis (Fig. 1, Table 1) showed terminal glucose, terminal heptose and 3-substituted glucose in equimolar amounts whereas the derivative of Kdo was not detected. The core OS was first dephosphorylated and then the ketone and carboxyl groups were reduced. Sugar analysis of this material showed the presence of glucose, heptose and 3-deoxyoctitol in the molar ratio of 2:3:1, and the following methylation analysis revealed the presence of terminal glucose and heptose, 3-substituted glucose, 3-substituted heptose, 3,7-di-substituted heptose and 5-substituted 3-deoxyoctitol residues.

To obtain sequence information on the core hexasaccharide, dephosphorylated OS was oxidized with sodium metaperiodate, reduced

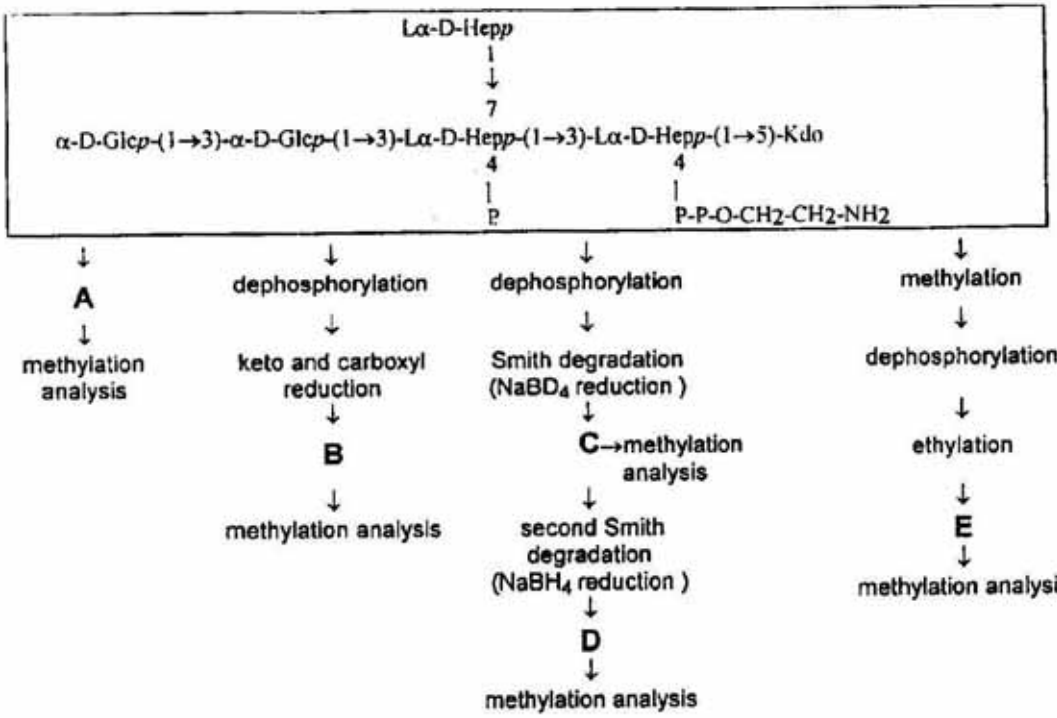

Fig. 1. Specific modifications and degradations of H. alvei core oligosaccharide.

with sodium borodeuteride, and hydrolysed with 2% acetic acid. Methylation analysis of the Smith degraded OS gave terminal glucose, 3-substituted 6-deuteromannose and 3-substituted heptose. The Smith-degraded OS was further oxidized with sodium metaperiodate, reduced with sodium borohydride and hydrolysed with acetic acid. Methylation analysis of the twice Smith-degraded material gave terminal mannose and 3-substituted 6-deuteromannose.

To obtain further sequence information, the core OS was analysed by the NOESY procedure. Inter-residue nuclear Overhauser effects, observed as cross-peaks, were found between

Table 1

Methylation analysis of original and modified core oligosaccharide (OS) from H. alvei strain 744 lipopolysaccharide.

For descriptions A through E see Fig. 1.

Sugar[a]	t_R[b]	Molar ratio				
		A	B	C	D	E
2,3,4,6-Glc	1.00	1.0	1.0	1.0	–[c]	1.0
2,3,4,6-Man	1.02	–	–	–	1.1	–
2,4,6-Glc	1.21	1.1	0.9	–	–	1.2
2,4,6-Man-6dd	1.25	–	–	1.0	1.0	–
1,2,4,6,7,8-Kdo-ol[e]	1.26	–	0.5	–	–	–
2,3,4,6,7-Hep	1.40	0.9	0.9	–	–	0.9
2,4,6,7-Hep	1.62	–	1.0	0.9	–	–
4-Et-2,6,7-Hep[f]	1.64	–	–	–	–	0.4
2,4,6-Hep	1.90	–	0.9	–	–	–
4-Et-2,6-Hep[g]	1.92	–	–	–	–	0.8

[a]2,3,4,6-Glc = 2,3,4,6-tetra-*O*-methyl-D-glucose, etc. [b]Retention time for the corresponding alditol acetate, relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, on an HP-1 glass capillary column at 150–270°C, 8°/min. [c]The dash indicates lack of the component. [d]6-Deutero-2,4,6-tri-*O*-methyl-D-mannose. [e]Keto and carboxyl reduced Kdo. [f]4-*O*-Ethyl-2,6,7-tri-*O*-methyl-L,D-heptose. [g]4-*O*-Ethyl-2,6-di-*O*-methyl-L,D-heptose.

H-1 of α-D-Glc-(1 → and H-3 of → 3)-α-D-Glc-(1 → (δ 5.37/3.94), H-1 of → 3)-α-D-Glc-(1 → and H-3 of → 3,7)-Lα-D-Hep-(1 → (δ 5.19/4.11), H-1 of → 3,7)-Lα-D-Hep-(1 → and H-3 of → 3)-Lα-D-Hep-(1 → (δ 5.10/4.06), H-1 of → 3)-Lα-D-Hep-(1 → and H-5 of → 5)-Kdo (δ 5.20/4.16) and between H-1 of Lα-D-Hep (δ 4.99) and a proton giving a signal at δ3.74, probably a non-assigned H-7.

The substitution positions of the phosphate groups in the core OS were determined by the methylation analysis involving methylation, removal of the phosphate groups using 48% hydrofluoric acid, followed by ethylation of the formed hydroxyl groups. By this procedure the hydroxyls carrying phosphate groups in the native oligosaccharide were labelled with *O*-ethyl groups. GLC-MS analysis gave, in addition to terminal glucose, terminal heptose, 3-substituted glucose and 5-substituted 3-deoxyoctitol, 3-substituted heptose with a 4-*O*-ethyl group and 3,7-di-substituted heptose with a 4-*O*-ethyl group. The phosphate substitution pattern was revealed from the inverse-detected H,P-correlation experiment (HMQC). This experiment and the ^{1}H NMR spectrum showed that the major part of the →3)-Hep-(1 → residue, with the anomeric proton signal at δ5.20, was substituted in the 4-position with a pyrophosphorylethanolamine group. This was evident from the phosphorus signals at δ –10.4 and –11.1 for pyrophosphate groups with P,H-connectivities to H-4 of the → 3)Hep-(1 → and H-1 of the ethanolamine residue, respectively. The → 3,7)-Hep-(1 → residue was substituted by a phosphate group in the 4-position. This was evident from the phosphorus signal at δ 1.2 with a P,H-connectivity to H-4 of the sugar residue.

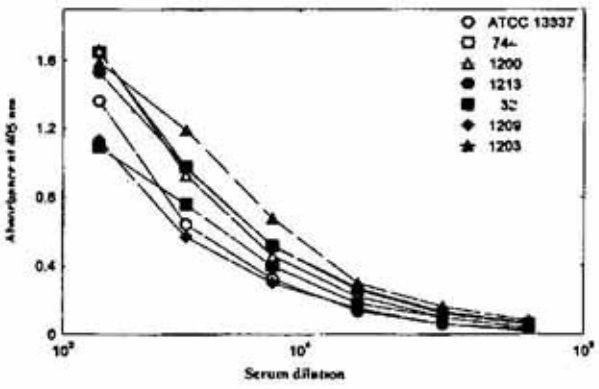

Fig. 2. Titration of anti-ATCC 13337 OS-TT serum in the enzyme-linked immunosorbent assay (ELISA) against different antigens.
The wells were coated with *H. alvei* lipopolysaccharides (10 μg/ml).

The results from sugar and methylation analysis together with NMR spectral data suggest that the core hexasaccharide present in lipopolysaccharides of *Hafnia alvei* strains: 32, 744, 1200, 1203, 1209 and 1213 has the structure shown in Fig. 1.

All lipopolysaccharides tested reacted strongly with anti-ATCC 13337 OS core-tetanus toxoid conjugate serum in ELISA test (Fig. 2). These cross-reactions indicated that the core region present in ATCC 13337 standard strain is a common structure in the *Hafnia* genus. This conclusion is a strong confirmation of our data concerning the structure of the *H. alvei* LPS core region.

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