Structural studies on the short-chain lipopolysaccharide of *Vibrio cholerae* O139 Bengal

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A *Vibrio cholerae* O139 strain, MO10-T4, lacking capsular polysaccharide, produces a short-chain lipopolysaccharide (LPS), similar to enterobacterial SR strains. It was studied by acidic and alkaline degradation, dephosphorylation, sugar and methylation analysis, high-performance anion-exchange chromatography, one- and two-dimensional 'H-, 'C-, and 31P-NMR spectroscopy, and electrospray ionization mass spectrometry. The following structure was proposed for the core region of the LPS:

![Structure of core region](image)

where PEtn stands for 2-aminoethyl phosphate, Fru for fructose, Hep for t-glucero-D-manno-heptose, and Kdo for 3-deoxy-D-manno-octulosonic acid; unless otherwise stated, the monosaccharide residues are D and present in the pyranose form. An O-acetyl group is present on a secondary position, tentatively on O4 of the α-linked glucosyl group. Some LPS species contain an additional putative fructose residue whose location remains unknown.

An O139-negative mutant strain, Bengal-2R, derived from *V. cholerae* O139, has also been investigated and shown to produce an O-antigen-lacking LPS similar to those from enterobacterial R strains, some of the LPS species containing the same core region as the strain MO10-T4 LPS and the other lacking the lateral heptose residue. The carbohydrate backbone core structure is the same for the *V. cholerae* O139 and *V. cholerae* O1 LPS, thus confirming the close relation between these bacteria; however, the 2-aminoethyl phosphate, the O-acetyl group, and the second fructose residue have not been reported for the O1 LPS. In the *V. cholerae* O139 strain MO10-T4 LPS, a short O-side chain is attached at position 3 of the 7-substituted heptose residue and has the same structure as one repeating unit of the *V. cholerae* O139 capsular polysaccharide. Some details of the structure proposed are at variance with those recently published for another *V. cholerae* O139 strain [Cox, A. D., Brisson, J.-R., Varma, V. & Perry, M. B. (1996) *Carbohydr. Res.* 290, 43–58; Cox, A. D. & Perry, M. B. (1996) *Carbohydr. Res.* 290, 59–65.]

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Abbreviations. Col, 3,6-dideoxy-L-xylo-hexose (colitose); CPS, capsular polysaccharide; CSF-MS, electrospray ionization mass spectrometry; Fru, fructose; GalA, galacturonic acid; GlcN-o1, 2-amino-2-deoxy-glucitol; GPC, gel permeation chromatography; Hep, t-glucero-D-manno-heptose; HMBC, heteronuclear multiple-bond correlation; HPAEC, high-performance anion-exchange chromatography; HSQC, heteronuclear single-quantum coherence; Kdo, 3-deoxy-D-manno-octulosonic acid; anKdo, anhydro Kdo; LPS, lipopolysaccharide; Etn, 2-aminoethanol (ethanolamine); PEtn, 2-aminoethyl phosphate; QuiNAc, 2-acetamido-2,6-dideoxyglucose (N-acetylmuramic acid), Sug, L-threo-hex-4-enuronic acid.
Until a new epidemic of diarrhoea started in India and South Bangladesh in late 1992, *Vibrio cholerae* non-O1 were not considered as epidemic agents of cholera. The new causative agent belonged to none of the then known 138 0-serogroups of *V. cholerae* and, hence, was named *V. cholerae* O139 with the synonym Bengal [1]. It shares several properties with and in many aspects is indistinguishable from *V. cholerae* O1 El Tor [2, 3]. The main difference between the O1 and O139 serogroups is the architecture of the cell wall. None of the *V. cholerae* O1 strains is encapsulated. Outer-membrane lipopolysaccharide (LPS) of both O1 serotypes, Inaba and Ogawa, have a long O-side chain identified as a homopolymer of 4-(3-deoxy-L-glycerol-tetraamido)-4,6-dideoxy-D-mannose [4, 5] terminated in the latter serotype with the 2-O-methyl derivative of the same monosaccharide [6]. In contrast, *V. cholerae* O139 produces a polysaccharide capsule [2, 7] and has an LPS with a short O-side chain [7-9]. Serological and genetic studies suggested that in *V. cholerae* O139 the capsular polysaccharide (CPS) has the same repeating structure, while glucose, fructose, L-glycero-D-manno-heptose, and 3-amino-2-deoxy-D-glucose, 2-amino-2,6-dideoxy-D-glucose, 2-amino-2,6-dideoxy-D-glucose, and D-galacturonic acid, which glucuronic, fructose, L-glycero-D-manno-heptose, and 3-deoxy-D-manno-octulosonic acid are present in the LPS only [7, 8, 14-17]. The following structure of the repeating unit of *V. cholerae* O139 CPS has been recently elucidated [16, 17], an unusual feature being the presence of a cyclic phosphate group:

\[
\begin{align*}
\alpha-\text{Col} & \rightarrow (1 \rightarrow 2) 4,6-P-\beta-D-\text{Galp} \\
& \rightarrow 1 \\
& \rightarrow 3 \\
& \rightarrow (1 \rightarrow 4) \alpha-D-\text{Galp} & \rightarrow (1 \rightarrow 3) \alpha-D-\text{QuipNAc} & \rightarrow (1 \rightarrow 4) \\
& \rightarrow 1 \\
& \rightarrow 1 \\
& \alpha-\text{Colp}
\end{align*}
\]

We now report structural characterization, including data on both the O-side chain and the core-lipid A region, of the LPS from *V. cholerae* O139. The preliminary results were presented at the XVIIIth International Carbohydrate Symposium (Milan, July 1996) [18]. When this work was in progress, the structure of the LPS from another *V. cholerae* O139 strain, NRCC 4740, was reported [19, 20], which differs in some details from the structure presented by us. Here, we discuss these differences as well as the similarity and difference between the *V. cholerae* O139 and *V. cholerae* O1 LPS.

**EXPERIMENTAL PROCEDURES**

**Bacteria, cultivation, and isolation of LPS.** Two mutant strains derived from *V. cholerae* O139 were used: MO10-T4, a capsule-free spontaneous translucent variant of the O139 opaque wild-type MO10, and Bengal-2R, an O139-negative transposon Tn5lac insertion mutant of an opaque vaccine derivative (Bengal-2) of MO10 [11]. Both strains were obtained from the Department of Microbiology and Molecular Genetics, Harvard Medical School (Boston MA, USA; Professors M. K. Waldor and J. J. Mekalanos). The bacteria were grown in a 30-l fermentor (Belach AB) in a rich tryptone/yeast extract as described earlier [7, 21]. Pelleted bacterial cells were suspended in water and extracted with hot aqueous phenol [22]. Crude LPS preparations were recovered from the aqueous phase by dialysis and, if necessary, purified by treatment with nucleases and proteases.

**Chromatography and mass spectrometry.** Gel-permeation chromatography (GPC) was performed on a column (75x2.6 cm) of Sephadex G-50 or a column (40x2.6 cm) of Bio-Gel P-2 using 0.05 M pyridinium acetate pH 4.5 as eluent, monitored with a Waters differential refractometer. High-performance anion-exchange chromatography (HPAEC) was performed on a Dionex Series 4500 chromatography system equipped with a semi-preparative column (9x250 mm) of CarboPac PA1 at 5 ml/min in 0.1 M sodium hydroxide, using a linear gradient of 2-100% 1 M sodium acetate in 30 min.

GLC was performed using a Hewlett-Packard 5890 instrument on a DB-5 fused-silica capillary column using a temperature gradient of 160°C (1 min) to 250°C at 3°C/min. GLC/MS was performed with the same type of chromatograph using a DB-1 column, connected to a Hewlett-Packard 5970 mass spectrometer, using the above conditions.

Electrospray ionization mass spectrometry (ESI-MS) was performed in the negative mode using a VG Quattro triple quadrupole mass spectrometer (Micromass) with aqueous 50% acetonitrile containing 1 mM ammonia as the mobile phase at a flow rate 10 μl/min; the samples were dissolved in aqueous 50% acetonitrile at a concentration of ~50 pmol/μl, and 10 μl was injected via a syringe pump into the electrospray source. Mass numbers were rounded to nearest integer.

**NMR spectroscopy.** NMR spectra of D2O solutions were run with Jeol EX-270, Varian Unity 500 or Varian Inova 600 spectrometers at 25°C, 43°C or 60°C and pH 3.5. Chemical shifts are reported in ppm, using internal sodium 3-trimethylsilyl[(2,2,3,3,4,4,5,5)2-H]propanoate (δH, 0.00), external deoxolane (δδ, 67.40) or external 2% phosphoric acid in D2O (δδ, 0.00 ppm) as references. Two-dimensional COSY, TOCSY, NOESY, ROESY, 'H-detected 'H,13C heteronuclear single-quantum coherence (HSQC), heteronuclear multiple-bond correlation (HMBC), and H-detected 13C COSY experiments were performed using standard pulse sequences. The TOCSY and ROESY experiments used mixing times of 10–100 ms and 350 ms, respectively. The delay time in the HMBC experiment was set to 50 ms.

**Sugar and 2-aminoethanol analyses.** Hydrolysis was performed with 2 M trifluoroacetic acid (100°C, 5 h), neutral sugars and amino sugars were identified by GLC as their alditol acetates [23] by comparison with authentic samples; relative ratios of monosaccharides were estimated directly from the detector response. Uronic acid was identified by GLC after methanolysis with 1 M hydrogen chloride in methanol (80°C, 16 h) and acetylation. Fructose was determined as a mixture of mannitol and glucitol obtained after hydrolysis with 2% acetic acid (100°C, 4 h) and sodium borohydride reduction. Amino sugars were N-acetylated with acetic anhydride in saturated aqueous sodium hydrogen carbonate solution as described [24]. Amino sugars and 2-aminoethanol were detected using a Biotronik LC-2000 amino acid analyzer, a column of a Qiong LN AN B cation-exchange resin, and 0.35 M sodium citrate, pH 5.28 at 80°C. Absolute configurations of monosaccharides were determined by GLC of the acetylated (+)-2-butyl glycosides (for GalA and GlcNAc) or acetylated (+)-2-octyl glycosides (for Col, Gal, and QuiA) [25, 26].

**Methylation analysis.** Sugars were methylated with methyl iodide in dimethyl sulfoxide in the presence of lithium methylsulfonyl methaneide [27] and the methylated compounds were recovered using a SepPak C18 cartridge. Methylated polysaccharides were hydrolysed as described for sugar analysis, the par-
tially methylated monosaccharides reduced with sodium borohydride, acetylated, and analyzed by GLC/MS. They were identified by comparison with published data [28, 29].

**Hydrazinolysis.** The LPS (700 mg) was treated with anhydrous hydrazine (15 ml) for 1 h at 37°C and then for 16 h at 20°C. The solution was poured into cold acetone and diethyl ether was added for better precipitation. The precipitate was separated by centrifugation, washed twice with acetone, and dried in air to give the O-deacylated LPS. Typical yields were around 80%.

**Deacylation.** LPS were dephosphorylated with aqueous 48% hydrofluoric acid at 4°C for 20 h. The solution was then cooled to −18°C and neutralized with equally cold aqueous 25% ammonia, and centrifuged. The supernatant was desalted by GPC on a column of Sephadex G-50 (for the acid-degraded LPS) or by dialysis against distilled water (for the O-deacetylated LPS). Typical yields were in the range 85–90%.

**Alkaline hydrolysis.** Protocol A. The O-deacylated and dephosphorylated LPS (200 mg) was reduced with sodium borohydride and dissolved in 4 M potassium hydroxide (5 ml). The solution was flushed with nitrogen for 30 min with stirring, heated for 8 h at 100°C, cooled, neutralized with 8 M hydrochloric acid, extracted twice with dichlormethane, and the aqueous solution was desalted by GPC on Sephadex G-50. The isolated product was then N-acetylated [24], eventual O-acetyl groups removed with aqueous 12% ammonia (50°C, 1 h), and fractionated by HPAEC. Fractions were desalted with IRA-120 cation-exchange resin (H+). The retention times of the fractions from the LPS of strain MO10-T4 were 6.98 min for T4-A1 and 10.26 min for T4-A2; that from strain Bengal-2R (2R-A1) was 6.98 min. Typical yields were in the range 20–25%, and the yields of individual fractions 2–4%.

**Protocol B.** The O-deacylated LPS (300 mg) was treated with 4 M potassium hydroxide, worked up as described above, and the product fractionated by HPAEC. The retention times of the fractions from the LPS from strain Bengal-2R were 16.95 min for 2R-B1, 17.17 min for 2R-B2, 18.32 min for 2R-B3, 19.39 min for 2R-B4; from strain MO10-T4, 20.33 min for T4-B1, 20.69 min for T4-B2, 21.64 min for T4-B3. Typical yields were in the range 25–30% and the yields of individual fractions 2–4%. The low yields can, at least partly, be attributed to the fact that the LPS is not purified.

**Acidic delipidation.** Protocol C. The LPS (300 mg) was dissolved in 0.1 M sodium acetate pH 4.2 (10 ml) and heated for 4 h at 100°C. The precipitate was removed by centrifugation, and the oligosaccharides isolated by GPC first on Sephadex G-50 followed by Bio-Gel P-2. Typical yields were in the range 15–20%.

**RESULTS**

The purified LPS from two V. cholerae O139-derived strains, MO10-T4 and Bengal-2R, was deacylated according to protocols A and B or delipidated by protocol C as shown in Fig. 1.

**Alkaline degradation of the dephosphorylated LPS.** LPS were subjected to hydrazinolysis at 37°C followed by dephosphorylation with 48% hydrofluoric acid, borohydride reduction, N-deacetylation by hydrazinolysis with 4 M potassium hydroxide at 100°C, and N-acetylation (protocol A) [30]. Preparative HPAEC on CarboPac PA1 gave two major fractions, T4-A1 and T4-A2, from the strain MO10-T4 LPS and only one fraction, 2R-A1, from the strain Bengal-2R LPS, which had the same retention time as T4-A1.

Sugar and absolute configuration analysis of T4-A1 demonstrated the presence of D-Glc, L-glycero-D-manno-heptose (Hep), D-GlcN, and 2-amino-2-deoxyglucitol (GlcN-ol) in the ratios 1:1.8:0.8:0.3. ESI-MS performed in the negative mode revealed doubly and triply charged pseudomolecular ions for T4-A1 which corresponded to the molecular mass of 1942 Da. This was consistent with an oligosaccharide containing the following residues: two Glc, four Hep, two GlcNAc, one GlcNAc-ol, and one 3-deoxyoctulosonic acid (Kdo).

Methylation analysis of T4-A1 showed that both Glc residues, and one residue each of Hep and GlcNAc occupied terminal non-reducing positions. Of the other three Hep residues, one was 7-substituted, the second 2,6-disubstituted, and the third 3,4,6-trisubstituted. The GlcN and GlcNAc residues which build the lipid A backbone, were both 6-substituted.

The 1H-NMR spectrum of T4-A1 closely resembled that of 1 (see below) obtained from the LPS of V. cholerae O1 smooth (Inaba) and rough mutant (Ogawa) strains [31] by protocol A. The spectrum contained, inter alia, signals for a methylene group of Kdo (H) at δ 2.18 (H34 and 1.80 (H33), and for eight anomeric protons, including those for four Hep residues at δ 5.74 (D), 5.26 (B), 5.20 (M), and 5.11 (E), α-Glc at δ 5.22 (F), β-Glc at δ 4.65 (G), α-GlcNAc at δ 4.75 (A), and β-GlcNAc (I) at δ 4.53, using the same sugar labeling as for the V. cholerae O1 core [31].

These data show that T4-A1 has structure 1. Comparison using HPAEC between T4-A1 and 1 isolated from the V. cholerae O1 LPS [31] confirmed this.

Sugar analysis of T4-A2 revealed the same components as in T4-A1 and, in addition, 2-amino-2,6-dideoxy-d-glucose (QuiN). The molecular mass of 2287 Da determined by ESI-MS indicated that T4-A2 also contained one residue of L-threo-hex-4-enuronic acid (Sug). This sugar is known to be derived from a 4-substituted hexuronic acid by β-elimination during strong al-
Fig. 2. HPAEC chromatograms from the strong alkaline degradation according to protocol B of the LPS from *V. cholerae* strains Bengal-2R (A) and MO10-T4 (B). For fraction designations see text.

alkaline treatment (cf. published data, e.g. [32]). The two additional sugars, QuiNAc and Sug, absent from the LPS from the R-mutant strain Bengal-2R (see below), were evidently derived from the O-side chain of the strain MO10-T4 LPS. Methylation analysis of T4-A2 revealed, *inter alia*, 3-substituted QuiNAc and 3,7-disubstituted Hep, the latter appearing instead of 7-substituted Hep (B) found in T4-A1. Therefore, Sug is attached to QuiNAc at position 3, and the site of attachment of the O-side chain is position 3 of Hep B.

A study of fraction 2R-A1 obtained from the strain Bengal-2R LPS by sugar and methylation analysis and by ¹H-NMR spectroscopy showed that it was a mixture of decasaccharide I and nonasaccharide 3 lacking the terminal Hep residue (M). As judged by the ratio of methylated derivatives derived from 2,6-disubstituted Hep (D) in I and 2-substituted Hep in 3, the content of I in the mixture was approximately twice that of 3.

Alkaline degradation of the native LPS. O-Deacylated LPS were N-deacylated by strong alkaline hydrolysis and fractionated by HPAEC on CarboPac PA1 (protocol B) [24, 34]. The LPS from strain Bengal-2R gave four major fractions in comparable amounts (Fig. 2A). Sugar analysis and ¹H- and ³¹P-NMR data showed that, in addition to the core components present in T4-A1, all isolated products contained fructose and phosphate and, instead of GlcN-ol of the lipid A backbone, α-GlcNIP (K) was present (δH, 5.74, J1,2, 3.5 Hz, J10,15, 5.9 Hz).

ESI-MS of the fraction eluted first (2R-B1) revealed the presence of doubly and triply charged pseudomolecular ions for two oligosaccharides with the molecular masses of 2216 Da (major) and 2024 Da (minor) indicating a difference of one Hep residue with four Hep residues in the major and three in the minor product, and the presence in both oligosaccharides of one fructose residue and three phosphate groups. Accordingly, 2R-B1 displayed a ³¹P-NMR spectrum containing three signals for monophosphate groups of Kdo4P (H), β-GlcN4P (I), and α-GlcN1P (K) at δ -2.0, -0.2, and 0.1. The ¹H-NMR data for 2R-B1 were closely similar to those for oligosaccharide trisphos-
Fig. 3. The δ 4.3–6.0 region of the ¹H-NMR spectrum of fraction T4-B3 obtained by strong alkaline degradation according to protocol B of the LPS from *V. cholerae* strain MO10-T4 followed by HPAEC.

The second fraction (2R-B2) had a ¹H-NMR spectrum similar to that of 2R-B1. As judged by ESI-MS data, the molecular mass of 2R-B2 was 2378 Da, and, therefore, like oligosaccharides 4, it contains three phosphate groups and four heptose residues, but differs in the presence of an additional hexose residue (or equivalent by molecular mass). This is suggested to be a second fructose residue on the basis of that (a) it was cleaved off with 48% hydrofluoric acid (cf. compound 1) or by hydrolysis at pH 4.2 (see compounds 15 and 16 below); (b) no hexose other than fructose was detected on mild acid hydrolysis of the products obtained from the LPS by protocol B, and (c) this residue could not be traced in the ¹H-NMR spectrum. Location of this putative fructose residue was not determined.

The ESI mass spectra of the third and fourth fraction (2R-B3 and 2R-B4) were almost indistinguishable and showed that each was a mixture of oligosaccharides containing four phosphate groups. Of them, two major products included one or two fructose residues and four heptose residues and one minor product contained one fructose residue and three heptose residues (their molecular masses were 2296, 2458, and 2104 Da, respectively). In addition to the ³¹P signals present in the ³¹P-NMR spectra of 2R-B1 and 2R-B2, the spectra of 2R-B3 and 2R-B4 each contained an additional signal at δ 0.5 and −1.3 ppm, respectively.

The oligosaccharide tetrakisphosphates were studied by NMR spectroscopy using COSY, TOCSY, NOESY, and ¹H-detected ¹H,³¹P COSY spectroscopy at high field. It was found that they differ from the corresponding oligosaccharide trisphosphates in the presence of the fourth phosphate group only. In 2R-B4 this group was found to be at position 7 of the Kdo⁴P residue (H), as followed, in particular, from a downfield displacement of the signal for Kdo H₇ to δ 4.37, as compared with its position in Kdo⁴P at δ 3.7–3.8 [31, 32], and from the presence in the ¹H,³¹P COSY spectrum of a cross-peak Kdo H₇, KdoP₇ at δ 4.37/1.3. In 2R-B3 the fourth phosphate group is suggested to be located at position 8 of Kdo⁴P. Good separation in HPAEC of isomeric oligosaccharide tetrakisphosphates present in 2R-B4 and 2R-B3 is a remarkable but not new phenomenon; thus, a significant difference in elution time has been noted for two oligosaccharide trisphosphates containing either Kdo7P or Kdo8P which were derived by protocol B from the LPS from a mutant strain of *Escherichia coli* K12 [35].

Fig. 4. The region of doubly charged pseudomolecular ions [M-2H]⁺ in the negative-mode ESI-MS spectrum of T4-C obtained by acidic delipidation of the LPS from *V. cholerae* strain MO10-T4. Compound A has structure 13.
The data obtained showed that the oligosaccharide tetraakisphosphates with one fructose residue present in 2R-B3 have the structures \(6\) and \(7\) and those present in 2R-B4 the structures \(8\) and \(9\).

\[
\begin{array}{ccc}
\text{A} & \text{M} & \text{F} \\
\alpha-\text{GlcN} & R^1 & \alpha-\text{Glc} \\
1 & 1 & 1 & R^2 & R^3 & P \\
1 & 1 & 1 & | & | \\
7 & 6 & 6 & 7 & 8 & 4 \\
\end{array}
\]

\[
\begin{array}{ccc}
\text{B} & \text{D} & \text{E} \\
\alpha-\text{Hep}(1\rightarrow2)-\alpha-\text{Hep}(1\rightarrow3)-\alpha-\text{Hep}(1\rightarrow5)-\alpha-Kdo(2\rightarrow6)-\beta-\text{GlcN}(1\rightarrow6)-\alpha-\text{GlcN}(1\rightarrowP) \\
\end{array}
\]

\[
\begin{array}{ccc}
\text{U} & \text{Q} & \text{K} \\
\beta-\text{Sug}(1\rightarrow3)-\beta-\text{D-QuipNAc}(1\rightarrow3)-\alpha-\text{Hep}(1\rightarrow2)-\alpha-\text{Hep}(1\rightarrow3)-\alpha-\text{Hep}(1\rightarrow5)-\alpha-Kdo(2\rightarrow6)-\beta-\text{GlcN}(1\rightarrow6)-\alpha-\text{GlcN}(1\rightarrowP) \\
\end{array}
\]

\[
\begin{array}{ccc}
\text{Z} & \text{G} \\
\beta-\text{Fruf}(2\rightarrow6)-\beta-\text{Glc} \\
\end{array}
\]

In addition to the four major fractions, three minor fractions were isolated from the oligosaccharide mixture from the strain Bengal-2R LPS and found to be by-products lacking \(\alpha-\text{GlcN1P}\), which, thus, were derived by destruction of the lipid A backbone during strong alkaline treatment.

The LPS from strain MO10-T4 gave three major fractions (Fig. 2B). They were studied using sugar analysis, ESI-MS and NMR spectroscopy as above. All the products were found to contain the same O-side-chain-derived sugars (QuiN and \(\beta-1,\text{-three-hex-4-enuronic acid}\)) as fraction T4-A2 obtained from strain M010-T4 by protocol A and the same core lipid A backbone constituents as fractions 2R-B1 to 2R-B4 obtained by protocol B from strain Bengal-2R.

ESI-MS data showed that each of the three fractions from the strain MO10-T4 LPS was a mixture of two compounds with one or two fructose residues, the latter being predominant. No minor product lacking the fourth Hep residue (M) was present. T4-B1 included oligosaccharide triakisphosphates with the molecular masses 2519 and 2681 Da, and T4-B2 and T4-B3 oligosaccharide tetraakisphosphates with the molecular masses 2599 and 2761 Da. The \(^1H\)-NMR spectrum of T4-B3 (Fig. 3) contained, inter alia, characteristic signals at \(\delta 5.92\) (H4, \(\alpha\)), \(\delta 5.28\) (H3, \(\beta\)), \(\delta 4.28\) (H3, \(\beta\)), and \(\delta 1.40\) (H6, \(\alpha\)). No significant changes were observed for these signals in the \(^1H\)-NMR spectrum of T4-B1 and T4-B2, but for the H7 signal of Kdo4,7P, \(\delta (H)\) which shifted upfield.

Detailed studies of the oligosaccharides using COSY, TOCSY, and NOESY confirmed their structures as analogues of the corresponding products obtained from strain Bengal-2R and the linkage of QuiN of the O-side chain to position 3 of Hep B.

Therefore, it was concluded that the oligosaccharide with one fructose residue present in T4-B1 was a tridecasaccharide trisphosphate \(10\), and those present in T4-B2 and T4-B3 were tridecasaccharide tetraakisphosphates \(11\) and \(12\), respectively.

Acidic delipidation of LPS. Delipidation of the LPS from strain Bengal-2R by mild acid hydrolysis at pH 4.2 (protocol C) resulted in an oligosaccharide mixture (2R-C), which was isolated by GPC. Sugar analysis by GLC of derived alditol acetates resulted in an oligosaccharide mixture (2R-C), which was isolated by GPC. Sugar analysis by GLC of derived alditol acetates revealed the presence of Glc and Hep in the ratio 1:0.9. After \(N\)-acetylation of 2R-C and hydrolysis, GlcN was revealed and the relative content of Hep increased (the ratios Glc/Hep/GlcN were 1:1.4:0.2) and, hence, in 2R-C GlcN (A) has a free amino group, which prevented hydrolysis of the glycosidic linkage between GlcN and Hep B. This was confirmed by treatment of 2R-C with nitrous acid (deamination [36]) resulting in elimination of GlcN (A) and its conversion mainly into 2,5-anhydromannose.

Analysis of \(N\)-acyetylated 2R-C using an amino acid analyzer after complete acid hydrolysis revealed the presence of 2-aminoethanol (Etn, the ratio GlcN/Etn = 1:0.4). Fructose was present in trace amounts, evidently due to its removal under the acidic conditions of delipidation of the LPS. Under these conditions, Kdo lost the phosphate group at position 4 and was converted into multiple anhydro forms (anKdo, H) [30, 37].

Similar acidic delipidation of the LPS from strain MO10-T4 afforded another oligosaccharide mixture (T4-C), which, in addition to the core constituents present in 2R-C, contained components of the O-side chain: galactose, 3,6-dideoxy-\(\alpha\)-xylo-hexose (colitose, Col), QuiN, and galacturonic acid (GalA). As in the studies of the \(V\). \textit{cholerae} O139 capsular polysaccharide [17],...
galactose was detected in the hydrolysate only when T4-C was dephosphorylated prior to hydrolysis and, hence, it was phosphorilated. During dephosphorylation with aqueous 48% hydrofluoric acid, most Col was also cleaved from T4-C. After dephosphorylation and N-acetylation of T4-C, the sugars Glc, Hep, Gal, GlcN, and QuiN were found in the proportions 2.8:4.3:1.1:1.4:1 as determined by GLC and, therefore, only one O-side-chain repeating unit is attached to the LPS core. The absence of Col is a result of the low yield of Col observed for all hydrolysates.

The 13C-NMR spectra of 2R-C and T4-C contained, inter alia, signals for 2-aminoethyl phosphate (CH₃N at δ 41.2, Jₑₚₑ 8 Hz; CH₂O at δ 63.1, Jₑₑ 5 Hz; cf. published data, e.g. [38]), and an O-acetyl group (CH₃ at δ 21.5, CO δ ~175). In the 13C-NMR spectrum of T4-C, signals for the O-side-chain repeating unit were easily observed, inter alia, those for C3 and C6 of two Col residues at δ 33.9 and 16.5-16.7, respectively, C6 of QuiNAc and GalA at δ 17.8 and 174.0, respectively, and two N-acetyl groups of GlcNAc and QuiNAc (CH₃ at δ 23.3 and 23.6, CO δ ~175). In the 1H-NMR spectrum of N-acetylated and dephosphorylated T4-C, while terminal Col, 3-substituted QuiNAc, and 3,4-disubstituted GlcNAc were found in the native T4-C, 2,4,6-trisubstituted Gal being detected in the latter only after dephosphorylation of the methylated product. Methylation analysis of deaminated T4-C revealed 3-substituted Hep instead of 3,7-disubstituted Hep (B), and, therefore, B is substituted with A at position 7 and with the O-side chain at position 3.

The ESI mass spectrum of T4-C contained predominantly doubly charged pseudomolecular ions (Fig. 4). The major ion corresponded to oligosaccharide 13 with the molecular mass 2566 Da, which included the complete O-side chain with one cyclophosphate group and the complete core with one O-acetyl group. The molecular mass of 2671 Da calculated from another significant pseudomolecular ion could be assigned to dehydrated oligosaccharide 14 differing from oligosaccharide 13 in the presence of 2-aminoethyl phosphate (PEtn). Dehydration of underivatized oligosaccharides is not uncommon in ESI-MS (see e.g. published data [40]). In addition, the spectrum of T4-C contained minor pseudomolecular ions for oligosaccharides lacking one or two Col residues which were evidently eliminated during the acidic delipidation of the LPS. ESI-MS of N-acetylated and dephosphorylated T4-C revealed the presence of an oligosaccharide with the molecular mass 2286 Da derived by removal of phosphate groups and both Col residues.

An indication of the position of PEtn on Kdo residues followed from the structures of oligosaccharides 11 and 12. It has recently been shown that 2-aminoethanol is cleaved from Kdo7-PEtN during strong alkaline treatment and that partial phosphate migration from O7 to O8 occurs, while no migration takes place in Kdo7P or Kdo8P under the same conditions [35]. No information was available on the behaviour of Kdo8PEtN under alkaline conditions. From these observations we tentatively assign PEtn to position 7 of Kdo, though position 8 is not excluded.

From these data it is indicated that oligosaccharides 13 and 14 have the following structures:

<table>
<thead>
<tr>
<th>a-Colp-(1→2)-4,6-P-β-d-Galp-</th>
<th>a-GlcN</th>
<th>a-Hep</th>
<th>a-Glc</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>1</td>
<td>R</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>β-d-GlcNAc-(1→4)-α-d-GalpA-(1→3)-β-d-QuipNAc-(1→3)-α-Hep-(1→2)-α-Hep-(1→3)-α-Hep-(1→5)-anKdo</td>
<td>B</td>
<td>D</td>
<td>E</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+OAc</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ESI-MS for 2R-C revealed a number of weak singly and strongly doubly charged pseudomolecular ions, all corresponding to dehydrated PEtn-containing oligosaccharides. These major components were complete core oligosaccharides 15 and 16 with

acetyl group, which is located at a secondary position, tentatively O4 of the α-linked glucosyl group (F), as suggested from 1H and 13C chemical shifts together with connectivity data from TOCSY and HMBC spectra.
or without an O-acetyl group having the molecular masses 1639 and 1597 Da, respectively, and the corresponding oligosaccharides lacking Hep M. Of the latter, two oligosaccharides 17 and 18 with the molecular masses 1446 and 1404 Da were minor, while two major oligosaccharides had the molecular masses 1526 and 1484 Da that may be accounted for by the presence of an additional phosphate group. The position of PEtN in these products could be deduced by the same consideration as for oligosaccharides 13 and 14.

\[
\begin{array}{cccc}
A & M & F \\
\alpha-\text{GlcN} & R^1 & \alpha-\text{Glc} \\
1 & 1 & 1 & P\text{EtN} \\
1 & 1 & 1 & 7 \\
7 & 6 & 6 & 7 \\
\end{array}
\]

\[
\alpha-\text{Hep}-(1\to2)-\alpha-\text{Hep}-(1\to3)-\alpha-\text{Hep}-(1\to5)-\text{anKdo} \\
B & D & 4 & E \\
1 & 1 & 1 & H \\
G & \beta-\text{Glc} & & \\
15 & R^1 & = & \alpha-\text{Hep}, \text{and Ac} \\
16 & R^1 & = & \alpha-\text{Hep} \\
17 & R^1 & = & \text{H and Ac} \\
18 & R^1 & = & \text{H} \\
\]

Expected pseudomolecular ions for the complete core oligosaccharides with the molecular masses 1558 and 1516 Da and Hep-M-lacking oligosaccharides with the molecular masses 1365 and 1323 Da were observed in the ESI mass spectrum of N-acetylated and dephosphorylated 2R-C. The spectrum also contained minor peaks for Glc-lacking oligosaccharides with the molecular masses 1395 and 1353 Da, which were not detected in the spectra of the alkali-degraded LPS.

DISCUSSION

The present structural studies show that a mutant strain, Bengal-2R derived from V. cholerae O139, has an LPS similar to those from enterobacterial R strains. It has the complete core and lacks the O-side chain, while V. cholerae O139 strain MO10-T4 produces an LPS which contains both the core and a short O-side chain, as enterobacterial SR strains. The structure for the core carbohydrate backbone proposed for V. cholerae O139 is the same as in V. cholerae O1 LPS [31]. Thereby the genetic data demonstrating the close relation between these bacteria [13, 41] are confirmed. However, the 2-aminoethyl phosphate, the O-acetyl group, and the second fructose residue found in the V. cholerae O139 LPS, have not been reported for the O1 LPS.

With the similarities in the core region, the structures of the LPS O-side chains are markedly different in the two V. cholerae O-serogroups. While in V. cholerae O1 it is a homopolymer of 4-(3-deoxy-l-glycero-tetronamylo)-4,6-dideoxy-d-mannose with a monosaccharide repeating unit [4, 5], the V. cholerae O139 the LPS possesses a short O-side chain of a hexasaccharide containing, inter alia, two colitose residues and a cyclic phosphate group. This short O-side chain is linked to the core oligosaccharide at position 3 of a heptose residue, whereas the site of attachment of the O-side chain in the V. cholerae O1 LPS remains unknown.

In accordance with serological and genetic data [10–12], the structural analysis showed that the O-side chain of the V. cholerae O139 LPS has the same structure as one repeating unit of the V. cholerae O139 CPS [16, 17]. The finding that the O-side chain is linked to the core via QuiNac demonstrated the structure of the biological repeating unit of the CPS. It is worth noting that in the LPS the QuiNac residue has the same β configuration as it has when linking repeating units in the CPS.

Heterogeneity in the core region of the V. cholerae O139 LPS is associated with the fourth heptose residue (tentatively a fructose residue) and the 2-aminoethyl phosphate. The latter is located at position 7 of Kdo4P and, therefore, this is the first finding of a bisphosphorylated Kdo residue in bacterial LPS, while Kdo4P is common for Vibrionaceae and some other bacterial families and Kdo7P[EtN has been reported for some strains of Enterobacteriaceae [42]. In the mutant strain Bengal-2R LPS lacking the O-side chain, incomplete O-acetylation and incomplete glycosylation with the lateral heptose residue further contribute to the structural heterogeneity.

Some structural details proposed for the V. cholerae O139 strain MO10-T4 LPS studied in this work are at variance with those reported for another V. cholerae O139 strain NRCC 4740 [19, 20]. Thus, the attachment of the O-side chain to the core was reported to be at position 2 of a heptose residue but the present studies show position 3. The presence of three glucose residues in the core could not be verified but only two of them. Also, as with the V. cholerae O1 LPS, the 2-aminoethyl phosphate, the O-acetyl group, and the second fructose residue found in both the V. cholerae O139 strain MO10-T4 and mutant strain Bengal-2R LPS, have not been reported in the V. cholerae O139 strain NRCC 4740.

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