

## Cold temperature-induced modifications to the composition and structure of the lipopolysaccharide of *Yersinia pestis*

Yuriy A. Knirel,<sup>a,\*</sup> Buko Lindner,<sup>b</sup> Evgeny Vinogradov,<sup>a,†</sup> Rima Z. Shaikhutdinova,<sup>c</sup> Sof'ya N. Senchenkova,<sup>a</sup> Nina A. Kocharova,<sup>a</sup> Otto Holst,<sup>b</sup> Gerald B. Pier<sup>d</sup> and Andrey P. Anisimov<sup>c</sup>

<sup>a</sup>*N. D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Moscow 119991, Russia*

<sup>b</sup>*Research Center Borstel, Leibniz Center for Medicine and Biosciences, D-23845 Borstel, Germany*

<sup>c</sup>*State Research Center for Applied Microbiology, Obolensk, Moscow Region 142279, Russia*

<sup>d</sup>*Channing Laboratory, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA*

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**Abstract**—Following a report of variations in the lipopolysaccharide (LPS) structure of *Yersinia pestis* at mammalian (37 °C) and flea (25 °C) temperatures, a number of changes to the LPS structure were observed when the bacterium was cultivated at a temperature of winter-hibernating rodents (6 °C). In addition to one of the known *Y. pestis* LPS types, LPS of a new type was isolated from *Y. pestis* KM218 grown at 6 °C. The core of the latter differs in: (i) replacement of terminal galactose with terminal D-glycero-D-manno-heptose; (ii) phosphorylation of terminal oct-2-ulosonic acid with phosphoethanolamine; (iii) a lower content of GlcNAc, and; (iv) the absence of glycine; lipid A differs in the lack of any 4-amino-4-deoxyarabinose and presumably partial (di)oxygenation of a fatty acid(s). The data obtained suggest that cold temperature switches on an alternative mechanism of control of the synthesis of *Y. pestis* LPS.

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### 1. Introduction

The natural environmental temperatures for *Y. pestis*, the cause of bubonic and pneumonic plague, may vary from 0 to 42 °C. Significant variations in the lipopolysaccharide (LPS) structure were observed when the bacteria are cultivated at 25–28 or 37 °C, including alteration of terminal core monosaccharides [D-glycero-D-

manno-heptose (DDHep) vs D-galactose; 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) vs D-glycero-D-talo-octulosonic acid (Ko)]<sup>1</sup> and change in the content of 4-amino-4-deoxy-L-arabinose (L-Ara4N) and the degree of acylation in lipid A.<sup>1–3</sup> These variations were accompanied by alteration in the LPS bioactivity suggesting a role for overcoming the defense systems of both warm-blooded mammals (host) and cold-blooded insects (vector). We now studied the LPS structure in a *Y. pestis* strain grown at 6 °C (LPS-6) to mimic the conditions in animals during winter hibernation, and compared it with those of the LPS from the same strain cultivated at 25 and 37 °C (LPS-25 and LPS-37, respectively).

### 2. Results and discussion

LPS-6 was isolated by phenol/chloroform/light petroleum extraction from *Y. pestis* KM218 grown at 6 °C

**Abbreviations:** Ara4N, 4-amino-4-deoxyarabinose; DDHep, D-glycero-D-manno-heptose; LDHep, L-glycero-D-manno-heptose; ESI FTICR, electrospray ionization Fourier transform ion-cyclotron resonance; Kdo, 3-deoxy-D-manno-oct-2-ulosonic acid; Ko, D-glycero-D-talo-oct-2-ulosonic acid; LA, lipid A; LPS, lipopolysaccharide; EtNP, phosphoethanolamine; 12:0, 16:1, 3HO14:0, lauroyl, palmitoleoyl, 3-hydroxymyristoyl groups.

\* Corresponding author. Tel./fax: +7 095 1376148; e-mail: [knirel@ioc.ac.ru](mailto:knirel@ioc.ac.ru)

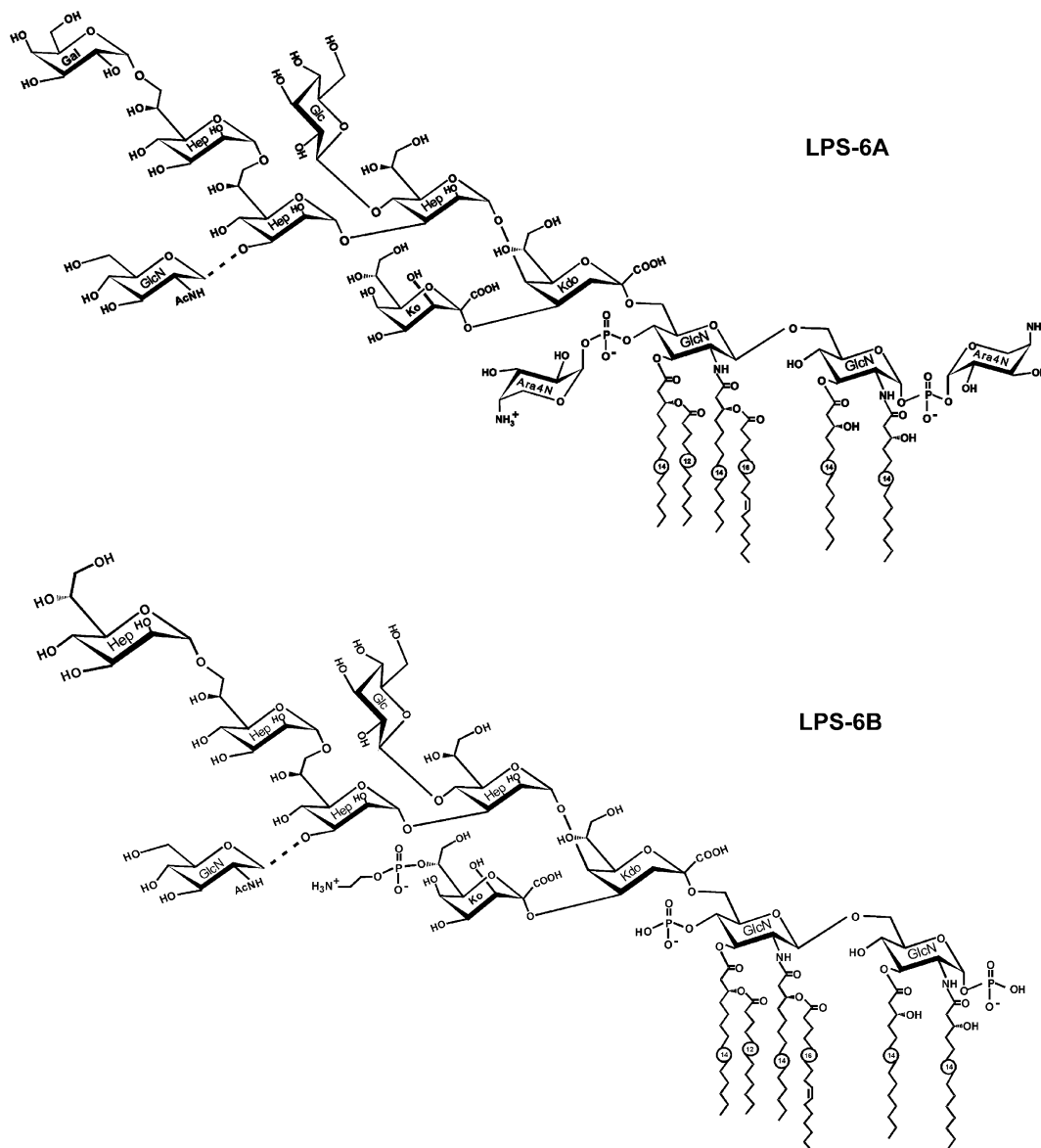
† Present address: Institute for Biological Sciences, National Research Council, Ottawa, ON, Canada K1A 0R6.

in a casein hydrolysate medium. In SDS-PAGE, the electrophoretic mobility of LPS-6 was practically the same as that for the previously described LPS-25<sup>1</sup> (data not shown).

The electrospray ionization Fourier transform ion-cyclotron resonance (ESI FTICR) mass spectrum of LPS-6 (not shown) indicated a mixture of two LPS types called LPS-6A and LPS-6B. The former was identified as one of the Ko-containing LPS-25 types with known structure<sup>1</sup> shown in Figure 1. Indeed, as in LPS-25, lipid A in LPS-6A bears four to six acyl groups and phosphate groups are substituted with 4-amino-4-deoxyara-

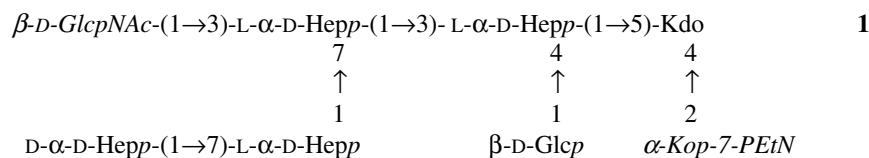
binose (Ara4N) nearly stoichiometrically. The core of LPS-6A contains only Gal and predominantly Ko as terminal core monosaccharides, which is in agreement with our previous finding that DDHep and Kdo are preferentially incorporated into the core at 37 °C, whereas Gal and Ko are incorporated at lower temperatures.<sup>1</sup>

In contrast, LPS-6B was significantly different from the known *Y. pestis* LPS types.<sup>1</sup> For the structural elucidation, LPS-6 was purified using the Bligh & Dyer procedure,<sup>4</sup> and LPS-6B was recovered from the chloroform/methanol/water extract. As from the ESI FTICR MS data, the LPS-6B preparation was



**Figure 1.** Structures of hexaacyl LPS-6A (LPS-6A<sub>hexa</sub>)<sup>1</sup> and hexaacyl LPS-6B (LPS-6B<sub>hexa</sub>) (this work) from *Y. pestis* KM218 cultivated at 6 °C. Pentaacyl LPS-6A lacks 16:1 and tetraacyl LPS-6A lacks both 16:1 and 12:0; tetraacyl LPS-6B lacks 16:1 and one of the 3-HO14:0 groups. In a minority of molecules of both LPS-6A and LPS-6B, terminal Ko is replaced with terminal Kdo. The dotted line indicates a non-stoichiometric substitution with GlcNAc (~50% in LPS-6A or ~15% in LPS-6B). Some molecules of LPS-6A contain glycine at an unknown position in the core. In lipid A of LPS-6B, an O-linked fatty acid(s) is partially (di)oxygenated.

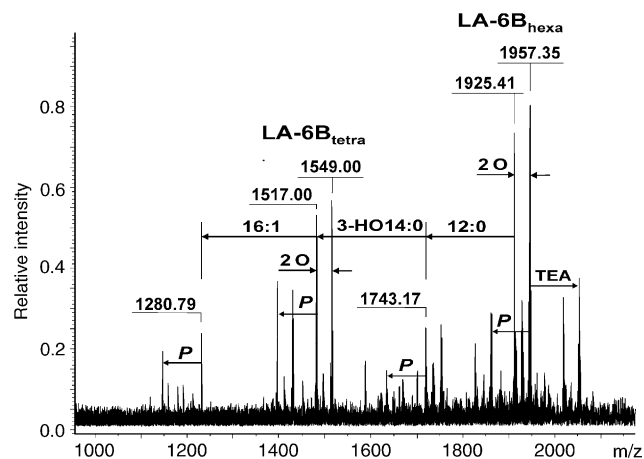




**Figure 4.** Structure of the core oligosaccharides isolated from LPS-6B. Ko, *D-glycero-D-talo*-oct-2-ulosonic acid, *D-α-D-Hep* and *L-α-D-Hep*, *D-glycero*- and *L-glycero-D-manno* heptose, *PEtN*, phosphoethanolamine. Non-stoichiometric constituents are shown in italics. In a minority of molecules, terminal *D-α-D-Hep* is replaced with terminal *β-D-Gal*.

$\delta_C$  75.4 from  $\delta_H$  4.11 and  $\delta_C$  72.2 in non-phosphorylated Ko (data of the 2D  $^1\text{H}$ ,  $^{13}\text{C}$  HSQC spectrum). The general structure of **1** shown in Figure 4 was confirmed by determination by ESI FTICR MS of the molecular masses of the LPS-6B-derived core oligosaccharides (Fig. 2), which differed from the calculated molecular masses by <0.03 Da.

The positive ion ESI FTICR mass spectrum of lipid A released by mild acid degradation of LPS-6B (Fig. 5) showed the major ion consistent with bisphosphoryl hexaacyl lipid A (LA-6B<sub>hexa</sub>,  $m/z$  1925.41) containing four 3-hydroxymyristoyl groups (3-HO14:0) and one group each of lauroyl (12:0) and palmitoleyl (16:1) groups.<sup>1,7</sup> Less intense was an ion for tetraacyl lipid A (LA-6B<sub>tetra</sub>,  $m/z$  1517.00) that lacks 12:0 and one of the 3-HO14:0 groups. There were also ions of minor intensity from pentaacyl and triacyl lipid A, which differed from LA-6B<sub>hexa</sub> and LA-6B<sub>tetra</sub> in the lack of 12:0 ( $\Delta m/z$  -182) and 16:1 ( $\Delta m/z$  -236), respectively, as well as monophosphoryl lipid A species resulting from the loss of one of the phosphate group ( $\Delta m/z$  -80) during mild acid degradation of LPS-6B. The assignment of the ions was in agreement with fatty acid analysis data of LPS-6B (Table 2). In addition, the spectrum contained ions consistent with the presence of tetra-



**Figure 5.** Positive ion ESI FTICR mass spectrum of lipid A isolated from LPS-6B. LA-6B<sub>hexa</sub> and LA-6B<sub>tetra</sub> stand for hexaacyl and tetraacyl lipid A. Given are mass numbers of monoadducts with triethylamine (TEA), which was added to improve the signal-to-noise ratio.

**Table 2.** GLC analysis of O-linked fatty acid composition of LPS-6B

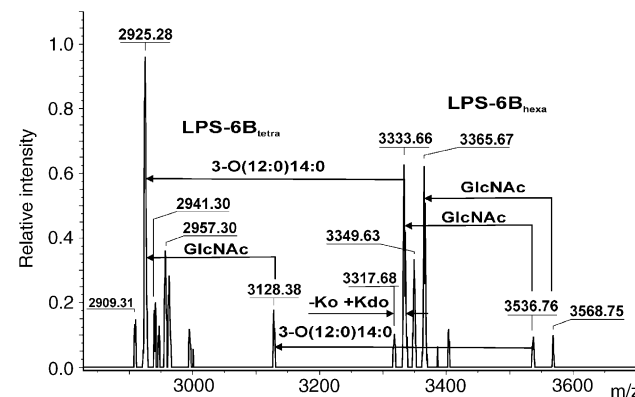
Fatty acid	Retention time (min)	Relative detector response
12:0	8.05	1.00
3-HO12:0 + 14:0	11.99	0.14
$\Delta^2$ 14:1 <sup>a</sup>	12.66	0.42
16:1	15.16	0.60
3-HO14:1	15.33	0.08
16:0	15.55	0.22
3-HO14:0	15.78	1.75
18:1	17.18	Trace
18:0	17.40	Trace

Given are data of the trimethylsilylated methyl esters of fatty acids released by alkaline hydrolysis of the LPS.

<sup>a</sup> From 3-HO14:0 by  $\beta$ -elimination of 12:0 or 16:1.

raacyl, pentaacyl, and hexaacyl lipid A species having a molecular mass higher by 32 Da than the corresponding counterparts with the defined composition (Fig. 5). These ions could reflect (di)oxygenation of an O-linked fatty acid(s); however, no major hydroxylated fatty acid other than 3-HO14:0 was detected in fatty acid analysis of LPS-6B. Therefore, the major ions at  $m/z$  1957.35 and 1549.00 must remain unassigned.

With the core and lipid A structures characterized, the ESI FTICR mass spectrum of the whole LPS-6B (Fig. 6) could be assigned. An ion at  $m/z$  3536.76 evidently belonged to hexaacyl LPS-6B with the core terminated



**Figure 6.** Negative ion ESI FTICR mass spectrum of the purified whole LPS-6B from *Yersinia pestis* KM218 cultivated at 6 °C. LPS-6B<sub>hexa</sub> and LPS-6B<sub>tetra</sub> stand for hexaacyl and tetraacyl LPS species. For the structure of LPS-6B<sub>hexa</sub>, see Figure 1B.

with one residue each of DDHep and EtNP-Ko as shown in Figure 1. Other compounds differ in the absence of GlcNAc ( $\Delta m/z -203$ ) and replacement of EtNP-Ko with EtNP-Kdo ( $\Delta m/z -16$ ) in the core as well as in the absence of 12:0 and one of the 3-HO14:0 groups from lipid A ( $\Delta m/z -408$ ). Neither ions for pentaacyl or triacyl lipid A species, nor those for phosphate- or EtNP-lacking molecules were observed in the mass spectrum of the whole LPS-6B, and, hence, in the isolated core and lipid A they were artifacts caused by mild acid degradation of the LPS. The absence of ions consistent with the presence of Gal-containing compounds (expected  $\Delta m/z -30$ ), which were detected as minor species in the isolated core (Fig. 2), could be accounted for by a too low content in intact LPS-6B. Again, there were ions of higher  $m/z$  values from dioxygenated compounds ( $\Delta m/z 32$ ), whose structures could not be determined.

Overall, cold temperature induced significant structural modifications in the LPS structure of *Y. pestis*. While LPS-6A possesses the same structure as the already determined structure of one of the LPS-25 types, LPS-6B is a new *Y. pestis* LPS type. The LPS-6B structure is distinguished by phosphorylation with phosphoethanolamine of terminal Ko or Kdo, whereas no core phosphorylation had been hitherto observed in any *Y. pestis* LPS. Another peculiar feature of LPS-6B is the complete lack of Ara4N in lipid A, which is a major component in other LPS types from strain KM218, including both LPS-25 and LPS-37, as well as in all other *Y. pestis* strains studied by us<sup>1</sup> and others<sup>2,3</sup> earlier. Remarkably, as opposed to LPS-6B, a high degree of substitution of lipid A phosphate groups with Ara4N was also observed in LPS-6A. Furthermore, LPS-6B is distinguished by the absence of glycine, a lower content of GlcNAc and replacement of Gal with DDHep in the core, oxygenation in lipid A and different fatty acid components of the tetraacyl species. These data suggest different mechanisms of control of the synthesis of LPS-6A (LPS-25, LPS-37) and LPS-6B. The latter switches on at cold temperatures, and one can speculate that the production of LPS-6B may be beneficial for the asymptomatic persistence of the bacterium in the host during winter hibernation. Comparative studies of bioactivity of LPS-6, LPS-25, and LPS-37 and structure–activity correlation of the *Y. pestis* LPS will be reported elsewhere.

### 3. Experimental

#### 3.1. Growth of bacteria, isolation of LPS, and SDS-PAGE

*Y. pestis* strain KM218, a plasmidless derivative of the Russian vaccine strain EV line NIEG, was grown at 6 °C in liquid aerated media containing fish-flour hydro-

lysate and yeast autolysate as described.<sup>1</sup> The lipopolysaccharide (LPS-6) was extracted from dried cells with phenol/CHCl<sub>3</sub>/light petroleum<sup>8</sup> and purified by enzymatic digestion of nucleic acids and proteins followed by repeated ultracentrifugation (105,000g, 4 h). LPS-6B was extracted from the LPS-6 preparation by the Bligh & Dyer procedure as described.<sup>4</sup> SDS-glycine polyacrylamide gel electrophoresis was performed and gels were silver stained as described.<sup>9</sup>

#### 3.2. Mild acid degradation of LPS

LPS-6 was degraded with aq 2% AcOH at 100 °C for 4 h, the water-insoluble crude lipid A precipitate (LA-6) was separated by centrifugation (13,000g, 15 min), washed with water, suspended in water, lyophilized, and the solid preparations were purified away from phospholipid contaminations by treatment with 1:1 CHCl<sub>3</sub>–MeOH.

The water-soluble supernatant was fractionated by GPC on a column (70 × 2.6 cm) of Sephadex G50 (S) (Amersham Biosciences, Sweden) using pyridinium acetate buffer (4 mL pyridine and 10 mL AcOH in 1 L water) as eluant and monitoring with a differential refractometer (Knauer, Germany), followed by anion-exchange chromatography on a HiTrap Q column (5 mL; Amersham Biosciences, Sweden) using water to elute neutral contaminants (10 min) and then a 0 → 1 M gradient of NaCl in water over 50 min to give two acidic fractions, which were desalted by GPC on Sephadex G-15. For NMR spectroscopic studies, the fraction eluted last was reduced with NaBH<sub>4</sub> in water (20 °C, 2 h).

#### 3.3. Fatty acid analysis

For O-linked fatty acid analysis, LPS-6 was saponified with 1 M NaOH (85 °C, 20 min), and then neutralized with 1 M HCl. Fatty acids were extracted with CHCl<sub>3</sub>, methylated with diazomethane, trimethylsilylated with *N,O*-bis(trimethylsilyl)trifluoroacetamide, and analyzed by GLC–MS on a Hewlett-Packard HP 5989A instrument equipped with a 30-m HP-5 ms column (Hewlett-Packard) using a temperature gradient of 150 °C (3 min) → 320 °C at 5 °C min<sup>-1</sup>.

#### 3.4. NMR spectroscopy

Prior to measurements, samples were exchanged twice with D<sub>2</sub>O. NMR spectra were recorded on a Varian Inova 500 spectrometer in D<sub>2</sub>O solns at 25 °C with acetone as an internal standard ( $\delta_{\text{H}}$  2.225,  $\delta_{\text{C}}$  31.5 ppm). Standard pulse sequences were used in 2D NMR experiments, including COSY, TOCSY (mixing time 120 ms), NOESY (400 ms), <sup>1</sup>H, <sup>13</sup>C gHSQC, <sup>1</sup>H, <sup>13</sup>C HMQC-TOCSY (100 ms), HMBC (100 ms), and <sup>1</sup>H, <sup>31</sup>P HMQC



(optimized for  $J_{P,H}$  11 Hz). Spectra were assigned using the computer program Pronto.<sup>10</sup>

### 3.5. Mass spectrometry

High-resolution ESI FTICR MS was performed on an ApexII-instrument (Bruker Daltonics, Billerica, USA) equipped with a 7 T actively shielded magnet and an Apollo electrospray ion source. Mass spectra were acquired using standard experimental sequences as provided by the manufacturer. For negative ion MS, samples ( $\sim 10 \text{ ng } \mu\text{L}^{-1}$ ) were dissolved in 50:50:0.001 2-propanol–water– $\text{Et}_3\text{N}$  (TEA) and sprayed at a flow rate of  $2 \mu\text{L min}^{-1}$ . When the whole LPS and lipid A were studied, TEA was added stepwise not to exceed pH 9 to avoid cleavage of O-linked fatty acids. For positive ion MS, a sample solution in 50:50:0.001 2-propanol–1 mM  $\text{AcONH}_4$ –TEA was adjusted to pH 4.5 with conc AcOH. Capillary entrance voltage was set to 3.8 kV and dry gas temperature to  $150^\circ\text{C}$ . The spectra were charge deconvoluted, and mass numbers given refer to the monoisotopic molecular masses.

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