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

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

a N. D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Moscow 119991, Russia
b Research Center Borstel, Leibniz Center for Medicine and Biosciences, D-23845 Borstel, Germany
c State Research Center for Applied Microbiology, Obolensk, Moscow Region 142279, Russia
d 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 DD-glycero-DD-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.

Keywords: *Yersinia pestis*; Lipopolysaccharide; Structure modification; Lipid A; Core oligosaccharide

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 alternation of terminal core monosaccharides [DD-glycero-DD-manno-heptose (DDHep) vs D-galactose; 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) vs D-glycero-D-talo-octulosonic acid (Ko)] and change in the content of 4-amino-4-deoxy-L-arabinose (L-Ara4N) and the degree of acylation in lipid A. 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...
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\(^1\) (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\(^1\) 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-deoxyarabinose (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.\(^1\)

In contrast, LPS-6B was significantly different from the known \textit{Y. pestis} LPS types.\(^1\) For the structural elucidation, LPS-6 was purified using the Bligh & Dyer procedure,\(^4\) and LPS-6B was recovered from the chloroform/methanol/water extract. As from the ESI FTICR MS data, the LPS-6B preparation was

\[\text{Figure 1. Structures of hexaacyl LPS-6A (LPS-6A_{hexa})}^1\] and hexaacyl LPS-6B (LPS-6B_{hexa}) (this work) from \textit{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)oxyxygenated.
essentially free from LPS-6A. LPS-6B was degraded under mild acid conditions to cleave the linkage between the core and lipid A moieties.

As opposed to the LPS-6A core, ions consistent with the presence of DDDDHep-containing compounds were abundant in the ESI FTICR mass spectrum of the core oligosaccharide mixture (Fig. 2), whereas ions consistent with Gal-containing oligosaccharides possessed only minor intensities. The spectrum showed structural heterogeneity owing to the presence or absence of GlcNAc ($\Delta m/z$ 203), Ko ($\Delta m/z$ 236), and/or phosphoethanolamine (EtNP, $\Delta m/z$ 123). The total content of GlcNAc in the core of LPS-6B was estimated as ~15% (compare ~50% in LPS-6A and ~90% in LPS-25 and LPS-371). In addition, Ko-containing molecules formed K- and Na-salts ($\Delta m/z$ 38 and 22, respectively), and Ko-lacking molecules occurred in both normal and anhydro forms ($\Delta m/z$ 18).

The Ko-containing and Ko-lacking oligosaccharides were separated by HiTrap Q anion-exchange chromatography, the former reduced with borohydride, and the structure of the major PETN-containing compound (I) was determined by $^1$H, $^{13}$C, and $^{31}$P NMR spectroscopy as described.5,6

Comparison of the NMR spectra of I and the corresponding oligosaccharide isolated earlier from another Ko-containing LPS-25 type1 showed that both have the identical carbohydrate backbone, the only distinction with I is the presence of EtNP. After assignment of the spectra (the key NMR chemical shifts are shown in Table 1), the location of EtNP at position 7 of terminal Ko was established by a 2D $^1$H, $^{31}$P HMQC experiment, which showed correlations of phosphorus at $\delta$ 0.9 with protons of ethanolamine at $\delta$ 4.19 (CH$_3$O) and $\delta$ 3.30 (CH$_2$N) as well as with H-7 of Ko at $\delta$ 4.52 (Fig. 3). This conclusion was confirmed by downfield displacements, due to a phosphate deshielding effect, of both H-7 and C-7 signals of EtNP-Ko to $\delta$H 4.52 and $\delta$C 74.5.

Table 1. NMR chemical shifts of EtNP-Ko in oligosaccharide I ($\delta$, ppm)

<table>
<thead>
<tr>
<th>Residue</th>
<th>Nucleus</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8a</th>
<th>8b</th>
</tr>
</thead>
</table>
| Ko      | $^1$H   | 4.05 | 4.02 | 4.15 | 3.89 | 4.52 | 3.96 | 4.06
|         | $^{13}$C | 72.8  | 66.9  | 68.6  | 71.7  | 75.4  | 62.0  |
| EtNP    | $^1$H   | 4.19  | 3.30  |       |       |       |       |     |     |
|         | $^{13}$C | 63.0  | 40.9  |       |       |       |       |     |     |
|         | $^{31}$P | 0.9  |       |       |       |       |       |     |     |
\[ \beta-D-GlcNAc-(1\rightarrow3)-L-\alpha-D-Hepp-(1\rightarrow3)-L-\alpha-D-Hepp-(1\rightarrow5)-Kdo \]

\( D-\alpha-D-Hepp-(1\rightarrow7)-L-\alpha-D-Hepp \)

\[ \beta-D-Glc \]

\[ \alpha-Kop-7-PEtN \]

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

\( \delta_C \) 75.4 from \( \delta_H \) 4.11 and \( \delta_C \) 72.2 in non-phosphorylated Ko (data of the 2D \( ^{1}H,{^13}C \) HSQC spectrum). The general structure of I 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\textsubscript{hexa}, \( m/z \) 1925.41) containing four 3-hydroxymyristoyl groups (3-HO14:0) and one group each of lauroyl (12:0) and palmitoleoyl (16:1) groups.\(^1\) Less intense was an ion for tetraacyl lipid A (LA-6B\textsubscript{tetra}, \( m/z \) 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\textsubscript{hexa} and LA-6B\textsubscript{tetra} 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 tetraacyl, 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.

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

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Retention time (min)</th>
<th>Relative detector response</th>
</tr>
</thead>
<tbody>
<tr>
<td>12:0</td>
<td>8.05</td>
<td>1.00</td>
</tr>
<tr>
<td>3-HO12:0 + 14:0</td>
<td>11.99</td>
<td>0.14</td>
</tr>
<tr>
<td>3(^\gamma)14:1*</td>
<td>12.66</td>
<td>0.42</td>
</tr>
<tr>
<td>16:1</td>
<td>15.16</td>
<td>0.60</td>
</tr>
<tr>
<td>3-HO14:1</td>
<td>15.33</td>
<td>0.08</td>
</tr>
<tr>
<td>16:0</td>
<td>15.55</td>
<td>0.22</td>
</tr>
<tr>
<td>3-HO14:0</td>
<td>15.78</td>
<td>1.75</td>
</tr>
<tr>
<td>18:1</td>
<td>17.18</td>
<td>Trace</td>
</tr>
<tr>
<td>18:0</td>
<td>17.40</td>
<td>Trace</td>
</tr>
</tbody>
</table>

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

* From 3-HO14:0 by \( \beta\)-elimination of 12:0 or 16:1.
with one residue each of DDHep and EtNP–Ko as shown in Figure 1. Other compounds differ in the absence of GlcNAc (Δm/z −203) and replacement of EtNP–Ko with EtNP–Kdo (Δm/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 (Δm/z −408). Neither ions for pentacyl 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 Δm/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 (Δm/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 and others 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 tetracyl 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 NIIEG, was grown at 6 °C in liquid aerated media containing fish-flour hydrolysate and yeast autolysate as described. The lipopolysaccharide (LPS-6) was extracted from dried cells with phenol/CHCl3/light petroleum 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. SDS-glycine polyacrylamide gel electrophoresis was performed and gels were silver stained as described.

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 (LPS-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 CHCl3–MeOH.

The water-soluble supernatant was fractionated by GPC on a column (70 × 2.6 cm) of Sephadex G50 (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 NaBH4 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 CHCl3, 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−1.

3.4. NMR spectroscopy

Prior to measurements, samples were exchanged twice with D2O. NMR spectra were recorded on a Varian Inova 500 spectrometer in D2O solns at 25 °C with acetone as an internal standard (δH 2.225, δC 31.5 ppm). Standard pulse sequences were used in 2D NMR experiments, including COSY, TOCSY (mixing time 120 ms), NOESY (400 ms), 1H,13C gHSQC, 1H,13C HMQC-TOCSY (100 ms), HMBC (100 ms), and 1H,31P HMOC.
Spectra were assigned using the computer program Pronto\textsuperscript{10}

### 3.5. Mass spectrometry

High-resolution ESI FTICR MS was performed on an Apex II 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 (~10 ng μL\textsuperscript{-1}) were dissolved in 50:50:0.001 2-propanol–water–Et\textsubscript{3}N (TEA) and sprayed at a flow rate of 2 μL min\textsuperscript{-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 AcONH\textsubscript{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 °C. The spectra were charge deconvoluted, and mass numbers given refer to the monoisotopic molecular masses.

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### References


