Temperature-Dependent Variations and Intraspecies Diversity of the Structure of the Lipopolysaccharide of *Yersinia pestis*<sup>1,‡</sup>


N. D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Moscow 119991, Russia, Research Center Borstel, Leibniz Center for Medicine and Biosciences, D-23845 Borstel, Germany, State Research Center for Applied Microbiology, Obolensk 142279, Moscow Region, Russia, Antiplague Research Institute of Siberia and Far East, Irkutsk 664047, Russia, and Channing Laboratory, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts 02115

Received July 22, 2004; Revised Manuscript Received October 18, 2004

**ABSTRACT:** *Yersinia pestis* spread throughout the Americas in the early 20th century, and it occurs predominantly as a single clone within this part of the world. However, within Eurasia and parts of Africa there is significant diversity among *Y. pestis* strains, which can be classified into different biovars (bv.) and/or subspecies (ssp.), with bv. orientalis/ssp. *pestis* most closely related to the American clone. To determine one aspect of the relatedness of these different *Y. pestis* isolates, the structure of the lipopolysaccharide (LPS) of four wild-type and one LPS-mutant Eurasian/African strains of *Y. pestis* was determined, evaluating effects of growth at mammalian (37 °C) or flea (25 °C) temperatures on the structure and composition of the core oligosaccharide and lipid A. In the wild-type clones of ssp. *pestis*, a single major core glycoform was synthesized at 37 °C whereas multiple core oligosaccharide glycoforms were produced at 25 °C. Structural differences occurred primarily in the terminal monosaccharides. Only tetraacyl lipid A was made at 37 °C, whereas at 25 °C additional pentaacyl and hexaacyl lipid A structures were produced. 4-Amino-4-deoxyarabinose levels in lipid A increased with lower growth temperatures or when bacteria were cultured in the presence of polymyxin B. In *Y. pestis* ssp. *caucasica*, the LPS core lacked D-glycero-D-manno-heptose and the content of 4-amino-4-deoxyarabinose showed no dependence on growth temperature, whereas the degree of acylation of the lipid A and the structure of the oligosaccharide core were temperature dependent. A spontaneous deep-rough LPS mutant strain possessed only a disaccharide core and a slightly variant lipid A. The diversity and differences in the structure of the *Y. pestis* LPS suggest important contributions of these variations to the pathogenesis of this organism, potentially related to innate and acquired immune recognition of *Y. pestis* and epidemiologic means to detect, classify, control and respond to *Y. pestis* infections.

Bubonic and pneumonic plague is caused by the Gram-negative bacterium *Yersinia pestis* circulating in natural foci in Eurasia, Africa, and the Americas, which involve a rodent reservoir and an insect vector (1–7). Endemic *Y. pestis* infection is due to continual transmission among susceptible rodents by various flea vectors, which results in acute infection with bacteremia in their enzootic rodent hosts. The high lethality of plague in rodent reservoirs is necessary for the organism’s continued transmission in nature. Fleas ingesting infected blood during preagonal bacteremia must depart from the host and feed on a new rodent, which subsequently becomes infected (1–7). It has been assumed that selective pressures within different host species and fleas contribute to the emergence of variant strains of *Y. pestis* which have been variously classified as biovars (bv.), based on differences in glycerol fermentation, nitrate reduction, and ammonia oxidation, or as subspecies (ssp.) or ecotypes (4), wherein strains are differentiated due to variations in fermentative activity, nutritional requirements, and ability to cause infectious bacteremia and death in diverse mammalian species.

The pathogenicity of *Y. pestis* is determined, in part, by a number of bacterial features that counteract mammalian (1–8) and insect (1, 2, 5–9) antimicrobial factors, ensuring...
maintenance of the pathogen in these hosts during the transmission cycle. One of these is the lipopolysaccharide (LPS), \(^1\) a virulence factor of many Gram-negative bacteria. LPS consists of three parts: lipid A, a core oligosaccharide, and an O-specific polysaccharide (O-antigen) whose synthesis gives rise to the smooth form of this structure. \(Y.\) \(pestis\) has a rough-type LPS (10–15) lacking the O-polysaccharide chain due to the nonfunctionality of the O-antigen gene cluster as a result of several frame-shift mutations (13, 14). In contrast, the enteropathogenic \(Yersinia\) species \(Yersinia\) \(pseudotuberculosis\) and \(Yersinia\) \(enterocolitica\), which cause chronic intestinal infections, possess a smooth-type LPS, which does not confer resistance to the bactericidal action of human serum (16) and antimicrobial peptides (17).

In mammals, the \(Y.\) \(pestis\) LPS mediates endotoxic shock via induction of potent cytokines whose production is balanced, in part, by the ability of the V antigen encoded on pCD to provoke production of the anti-inflammatory cytokine, interleukin-10 (IL-10) (18) as well as bind to toll-like receptor 2 which may provide a pro-inflammatory stimulus as well. Because IL-10 production, like that of most cytokines made during infection can be cyclic (19), it is likely that LPS-induced inflammatory cytokines significantly contribute to the host’s death when the effects of IL-10 are insufficient to counteract the effects of LPS (3, 20). LPS also plays a role in resistance of \(Y.\) \(pestis\) to serum-mediated lysis (16), which is necessary for survival and growth of the bacteria in mammalian blood (2–7, 9). However, as opposed to the majority of \(Y.\) \(pestis\) strains, some natural isolates of ssp. \(caucasica\) are highly susceptible to the bactericidal action of normal human serum (4), suggesting differences in the LPS structure of this variant \(Y.\) \(pestis\) ssp. The \(Y.\) \(pestis\) LPS structure also determines bacterial resistance to cationic antimicrobial peptides (17), a key component of innate immunity in both mammals and insects (21). Again, representatives of ssp. \(caucasica\), as well as those of ssp. \(hisarica\) and fresh isolates of ssp. \(altaica\), are highly sensitive to polymyxin B (4). \(Y.\) \(pestis\) strains belonging to all ssp. are highly lethal for mice and the overwhelming majority of ssp. \(pestis\) isolates are also lethal for guinea pigs, whereas strains of ssp. \(caucasica\), \(hisarica\), and \(altaica\) are usually of low virulence or even avirulent in guinea pigs (4).

Recently, the structure of the \(Y.\) \(pestis\) LPS has been extensively studied. The full lipid A structure (22, 23), a partial core structure of one strain (11), and preliminary data on the full structure of the LPS oligosaccharide from another strain (15, 24) have been published. The lipid A structure in \(Y.\) \(pestis\) bv. orientalis has been found to depend on the growth temperature and suggested to have biological significance in regard to transmission in fleas and mammals (23). However, no comprehensive analysis of the \(Y.\) \(pestis\) LPS structure, including both lipid A and substituent oligosaccharide structures synthesized by various \(Y.\) \(pestis\) strains differing in their geographical occurrence and epidemiologic significance has been reported. Here we describe results from studies of the structure of the LPS from strains of \(Y.\) \(pestis\) ssp. \(pestis\) (consisting of bvs. orientalis, antiqua, and mediavis) and ssp. \(caucasica\) (bv. antiqua) grown at different cultivation temperatures, which lead to distinct changes in the structure of this key pathogenic factor. In addition, we have characterized the LPS structure of a mutant strain EV11M, which is susceptible to complement and antimicrobial peptides in order to determine whether these biologic properties are associated with changes in the LPS structure.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains.** \(Y.\) \(pestis\) strain KIMD1 was kindly provided by Dr. M. Skurnik (University of Turku, Turku, Finland). Other \(Y.\) \(pestis\) strains used were obtained from the Russian Research Anti-Plague Institute “Microbe” (Saratov, Russia). Characteristics of the strains are given in Table 1. To guarantee the safety of the investigators, \(Y.\) \(pestis\) strains were attenuated by elimination of the virulence plasmid, pCD (7). None of the absent plasmids or missing parts of the genome contained genes for LPS biogenesis in any of the strains except for strain EV11M. All parental strains were virulent in both mice and guinea pigs, except for strain 1146, which is virulent in mice and avirulent in guinea pigs. Bacterial cultures were started from lyophilized stocks.

**Growth of Bacteria.** For LPS isolation, the strains were grown at 37 or 25 \(^\circ\)C in New Brunswick Scientific fermentors with working volumes up to 10 L of liquid aerated media. Growth medium was composed of fish-flour hydrolysate (20–30 g/L), yeast autolysate (10 g/L), glucose (3–9 g/L), \(K_2HPO_4\) (6 g/L), \(KH_2PO_4\) (3 g/L), and \(MgSO_4\) (0.5 g/L); pH 6.9–7.1. \(pH\) and \(pO_2\) control was used with a specified \(pO_2\) value >10%. For some LPS preparations, polymyxin B (AppiChem GmbH, Germany) was added to the nutrient medium to a final concentration of 20 \(\mu\)g/mL. Biomasses were harvested by centrifugation after 48 h of

### Table 1: \(Y.\) \(pestis\) Strains Used in These Studies

<table>
<thead>
<tr>
<th>Strain</th>
<th>Biovar/Subspecies</th>
<th>Relevant Characteristics</th>
<th>Geographical Origin of the Parent Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>KM218</td>
<td>orientalis/pestis</td>
<td>pFra, pCD; pPst; Δpgm; derived from the Russian vaccine strain EV line NIEEG</td>
<td>Madagascar</td>
</tr>
<tr>
<td>EV11M</td>
<td>antiqua/pestis</td>
<td>pFra, pCD; pPst; Δpgm; derived from the Russian vaccine strain EV line NIEEG and carrying undefined chromosomal mutation(s) resulting from multiple in vitro passages</td>
<td>Madagascar</td>
</tr>
<tr>
<td>KM260(11)</td>
<td>antiqua/pestis</td>
<td>pFra, pCD; pPst; derived from the virulent strain 231</td>
<td>Aksai focus, Kirghizia</td>
</tr>
<tr>
<td>K1146</td>
<td>antiqua/caucasia</td>
<td>pFra, pCD; pPst; derived from the virulent strain 1146</td>
<td>Trans-Caucasian-highland focus, Caucasus</td>
</tr>
</tbody>
</table>

\(^a\) For more detailed information on biovar-subspecies interrelations see ref. (4).
incubation and then lyopholized. Solid agar medium was prepared by adding 2% agar, pH 7.2 to the liquid medium.

Isolation of LPS and SDS—PAGE. LPS was extracted from dried cells with phenol/chloroform/light petroleum ether (25), enzymatically digested first with nucleases then proteases, and further purified by repeated ultracentrifugation (105,000g, 4 h). The purity of the isolated LPS preparations was evident from the lack of protein contamination assessed by gel electrophoresis and nucleic acid contamination as determined by sugar composition analysis (see below). A smooth-type LPS of *Escherichia coli* O55:B5 (Sigma) was used as a control in some assays. The LPS preparations extracted from different *Y. pestis* cultures grown at 25 °C or 37 °C are designated as LPS-25<sub>strain no.</sub> or LPS-37<sub>strain no.</sub> or, when appropriate, <sub>strain no.</sub>-25 or <sub>strain no.</sub>-37, respectively.

SDS–glycine polyacrylamide gel electrophoresis and silver staining of the gels were performed as described (13).

Mild Acid Degradation. LPS-25 and LPS-37 from each strain were degraded with aqueous 2% HOAc at 100 °C for 4 h. The water-insoluble lipid precipitates (crude lipid A) were separated by centrifugation (12000g, 15 min), washed with water, suspended in water, and lyophilized, and the solid preparations were then extracted with a chloroform–methanol mixture (1:1, v/v), which extracts free phospholipids from lipid A. The lipid A preparations are designated as LA-25<sub>strain no.</sub> and LA-37<sub>strain no.</sub> as per the designations for the initial LPS preparations.

The water-soluble supernatants from the wild-type-LPS strains *Y. pestis* KM218, KM260(11), KIMD1, and 1146 were fractionated by gel-permeation chromatography on a column of Sephadex G-50 (S) (70 × 2.6 cm; Amersham Biosciences, Sweden) using aqueous 1% HOAc supplemented with 0.4% pyridine as eluant. The supernatants from *Y. pestis* EV11M were fractionated by gel-permeation chromatography on a column of TSK HW-40 (S) (75 × 1.6 cm; Merck, Germany) in aqueous 1% HOAc. Monitoring was performed with a differential refractometer (Knauer, Germany). Fractions that contained core oligosaccharides (OSs) were collected and lyopholized. The OSs are designated as OS-25<sub>strain no.</sub> and OS-37<sub>strain no.</sub> similar to the designations of the initial LPS preparations.

Fractionation of the oligosaccharide from *Y. pestis* KM218 was achieved by anion-exchange chromatography on a HitTrap Q column (5 mL; Amersham Biosciences, Sweden) using water to elute neutral contaminants (fraction I) and then a 0 → 1 M gradient of NaCl in water to elute two fractions, designated II and III, which were subsequently desalted on Sephadex G-15. Fraction III from LPS-25 of *Y. pestis* KM218 was reduced by treatment with NaBD<sub>4</sub> in water and further fractionated by high-performance anion-exchange chromatography on a semi-preparative column of CarboPac PA100 (250 × 9 mm; Dionex, USA) in a 0.05 → 0.5 M gradient of NaOAc in 0.1 M NaOH at 3 mL/min over 1 h. Fractions of 3 mL were collected and analyzed on an analytical column of CarboPac PA100 (250 × 4.6 mm; Dionex, USA) using the same gradient run at 1 mL/min.

Composition and Methylation Linkage Analyses. Sugar analysis was performed by gas–liquid chromatographic (GLC) separation of monosaccharides and identification of the sugar monomers that had been derivitized to alditol acetates (26). Hydrolysis of the LPS oligosaccharide was performed with 2 M CF<sub>3</sub>CO<sub>2</sub>H (4 h, 100 °C) followed by conventional reduction with NaBH<sub>4</sub> and acetylation with Ace<sub>2</sub>O in pyridine (1:1.5, v/v; 100 °C, 30 min). GLC of the derived alditol acetates was carried out using a Hewlett-Packard 5880 chromatograph (Avondale, PA) equipped with a DB-5 fused-silica capillary column (30 m × 0.25 mm) and a temperature program of 160 °C (1 min) → 260 °C at 3 °C/min. Amino components were analyzed after hydrolysis with 4 M HCl (100 °C, 16 h), using a Biotronik LC-2000 amino acid analyzer equipped with a column (0.4 × 22 cm) of Ostion LG AN B cation-exchange resin in 0.2 M sodium citrate buffer, pH 3.25, at 80 °C.

For detection of 3-deoxy-d-manno-oct-2-ulonsic acid (Kdo), d-glycero-d-talo-oct-2-ulonsic acid (Ko) and 4-amino-4-deoxyarabinose (Ara4N), the acetylated methyl glycosides were prepared by methanolysis of the LPS with 2 M HCl in methanol (45 min or 16 h, 85 °C). After removal of the solvent the products were acetylated with Ac<sub>2</sub>O in pyridine (1:1.5, v/v, 85 °C, 20 min) and analyzed by GLC—MS on a Hewlett-Packard HP 5989A instrument equipped with a 30-m HP-5MS column (Hewlett-Packard) using a temperature gradient 150 °C (3 min) → 320 °C at 5 °C/min. Ammonia was used as reactant gas in chemical ionization MS.

For fatty acid analysis, lipid A was methanolized with 2 M HCl in methanol (85 °C, 16 h), and after evaporation of the solvent the product was acetylated with Ace<sub>2</sub>O in pyridine (1:1.5, v/v, 85 °C, 20 min) and analyzed by GLC—MS on a HP-5MS as described above.

For linkage analysis, the OS was methylated with CH<sub>3</sub>I in Me<sub>2</sub>SO in the presence of sodium methylsulfinylmethanide (27), and hydrolysis was performed as for the sugar analysis. The partially methylated monosaccharides were reduced with NaBD<sub>4</sub>, subsequently converted to the alditol acetates and analyzed by GLC—MS as described above.

NMR Spectroscopy. Prior to measurements, samples were exchanged twice with D<sub>2</sub>O. <sup>1</sup>H and <sup>13</sup>C NMR spectra were recorded on a Varian Inova 500 spectrometer in D<sub>2</sub>O solutions at 25 °C with acetone as an internal standard (δ<sub>H</sub> 2.225, δ<sub>C</sub> 31.5 ppm). Standard pulse sequences were used in two-dimensional NMR experiments, including COSY, TOCSY (mixing time 120 ms), ROESY (mixing time 250 ms), and <sup>1</sup>H, <sup>13</sup>C gHSQC. Spectra were assigned using the computer program Pronto (28).

Mass Spectrometry. High-resolution electrospray ionization Fourier transform ion cyclotron resonance (ESI FT—ICR) MS was performed in the negative ion mode using an ApexII-instrument (Bruker Daltonics, Billerica, MT) 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. Samples were dissolved at a concentration of ~10 ng/μL in a 50:50:0.001 (v/v/v) mixture of 2-propanol, water, and triethylamine and sprayed at a flow rate of 2 μL/min. Capillary entrance voltage was set to 3.8 kV, and dry gas temperature to 150 °C. Capillary skimmer dissociation (CSD) was induced by increasing the capillary exit voltage from −100 to −350 V. The spectra were charge deconvoluted, and the mass numbers given refer to the monoisotopic molecular masses.
RESULTS

Isolation and Characterization of the LPS from Y. pestis.
Each Y. pestis strain was cultivated at 25 and 37 °C, and the corresponding lipopolysaccharides (LPS-25 and LPS-37) were isolated by phenol/chloroform/light petroleum extraction and studied by SDS-PAGE (Figure 1). There were no significant distinctions in mobility of the LPS samples from Y. pestis ssp. pestis strains KM218, KM260(11), KIMD1 and Y. pestis ssp. caucasica strain 1146, but LPS preparations from strains grown at 37 °C migrated through the gel slightly faster and as more compact bands compared to the migration of LPS synthesized at 25 °C, suggesting that LPS-25 molecules are on average bigger than those of LPS-37.

The LPS-25 and LPS-37 from a known mutant strain, Y. pestis EV11M, derived by repeated serial passage from the same parental strain as KM218, migrated much faster in the SDS gels (Figure 1). This finding indicated that one effect of the mutations introduced into this strain resulted in a LPS with a smaller oligosaccharide compared with other Y. pestis strains.

GLC–MS of the acetylated derivatives obtained after methanolysis of the LPS from all strains studied revealed the presence of Kdo, Ko, and Ara4N. In addition, a derivative of a Ko–Kdo disaccharide was identified by the presence of a [M – CO2Me]⁺ ion at m/z 793 and typical fragmentation ions for Kdo at the reducing end (m/z 375) and Ko at the nonreducing end (m/z 461) in electron impact MS (29). The Ko–Kdo disaccharide structure was further confirmed by the presence of a [M + 18]⁺ ammonium-adduct ion at m/z 870 in chemical ionization MS (for the electron impact mass spectrum and explanation of diagnostic ions see Supporting Information).

Mass Spectrometric Analysis of the Oligosaccharides from Y. pestis ssp. pestis strain KM218.
The negative ion ESI FT–ICR mass spectrum of OS-37218 (Figure 2A) showed one major peak at m/z 1371.45, which was in excellent agreement with a heptasaccharide composed of GlcNAc(5)GlcHep(4)Kdo1 (compound 1a, calculated molecular mass 1371.45 Da). There was also a minor satellite peak at m/z 1353.45 (Δm/z −18) for the same compound indicating the presence of some Kdo in an anhydro from. A second minor peak (~10%) was present at m/z 1168.38 (Δm/z −203) that corresponded to compound 1a lacking GlcNAc (compound 1a′); it was thus estimated that GlcNAc is present in ~90% of the molecules. A third minor peak was present at m/z 1607.52 (Δm/z +236), indicating the presence of OS-37218 of molecules with an additional Ko residue (compound 1c).

The negative ion ESI FT–ICR mass spectrum of OS-25218 (Figure 2B) was more complex and showed four series of major ions for compounds 1a, 1b, 1c and 1d. The ion for 1a in the mass spectrum of OS-25218 had the same molecular
mass as in that of OS-37,218 (Figure 2A), indicating the presence of the same heptasaccharide. Another peak indicated the presence of a related hexasaccharide that lacked GlcNAc (compound 1a'), with a calculated degree of substitution of OS-25,218 with GlcNAc being ~65%. Ions of another series in OS-25,218 indicated the presence of a heptasaccharide, along with a hexasaccharide that was not found in OS-37,218 and differed from compounds 1a and 1a' by -30 Da. This structure had a replacement of one of the heptose residues (DD-Hep, see below) with Gal. Thus, OS-25,218 contains a heptasaccharide composed of GlcNAc,Glc,Hept,Kdo, (1b) and a hexasaccharide composed of Glc,Gal,Hept,Kdo, (1b'). Two remaining ions were detected in OS-25,218 that were characterized by a mass difference of +236 Da from ions 1a, 1a', 1b and 1b', evidently belonging to compounds with one additional Ko residue. They included peaks for two major octasaccharides GlcNAc,Glc,Hept,Kdo,Ko (1c) and GlcNAc,Glc,Gal,Hept,Kdo,Ko, (1d) with molecular masses 1607.6 and 1577.5 Da, and two minor GlcNAc-lacking heptasaccharides 1c' and 1d' (~203 Da), respectively.

Overall, these results indicate the occurrence of several LPS core oligosaccharide variants in *Y. pestis* ssp. *pestis* strain KM218, whose ratios vary significantly with growth temperature. Further determinations of the oligosaccharide structures from *Y. pestis* ssp. *pestis* were carried out on fractionated oligosaccharides OS-37,218 and OS-25,218 that were separated into differentially charged components by anion-exchange chromatography on a HiTrap Q column (Figure 3). OS-37,218 had only one major acidic fraction, II, whereas OS-25,218 yielded two acidic fractions, II and III, which corresponded to Ko-lacking and Ko-containing oligosaccharides, respectively. ESI FT−ICR MS showed that fraction II from OS-37,218 contains mainly oligosaccharide 1a with a molecular mass of 1371.45 Da (see Figure 2A), whereas fraction II from OS-25,218 is a mixture of oligosaccharides 1a and 1b with molecular masses of 1371.50 and 1341.49 Da and fraction III is a mixture of oligosaccharides 1c and 1d with molecular masses of 1607.55 and 1577.54 Da, respectively (see Figure 2B). Each fraction also contained the smaller-sized oligosaccharide lacking GlcNAc.
Analysis of the Oligosaccharides from Y. pestis KM260-(11) and KIMD1. To determine if there was a significant structural variability in the LPS oligosaccharides among different biovar strains of Y. pestis ssp. pestis, we next analyzed the oligosaccharides isolated from strains KM260(11) (bv. orientalis) and KIMD1 (bv. antiqua). Essentially the same oligosaccharide structures were found in OS-37 and OS-25 from these strains as was found in those from Y. pestis KM218. Strains KM260(11) and KIMD1 differed by having a slightly higher content of the Ko-containing compound in OS-37, which was present in only small amounts in OS-37 from strain KM218. Overall, the LPS oligosaccharide structures determined for three different strains of Y. pestis ssp. pestis were highly similar.

Linkage Analysis and Full Elucidation of the Structure of the Oligosaccharides from the LPS of Y. pestis KM218. Methylation of fraction III (Figure 3) from OS-25218 followed by hydrolysis and analysis by GLC-MS of the derived alditol acetates revealed 2,6,7-tri-O-methyl, 2,4,6-tri-O-methyl, and 2,3,4,6-tetra-O-methyl derivatives of LD-Hep indicating these methylated sugars were derived from 3,4-disubstituted, 3,7-disubstituted and 7-substituted LD-Hep residues. Additionally present was the 2,3,4,6,7-penta-O-methyl derivative of dd-Hep, 2,3,4,6-tetra-O-methylgalactose, and 2-amino-2-deoxy-2,3,4,6-tetra-N,O-methylglucose. As these methylated sugars had all of the available hydroxyl groups methylated, they indicate the presence of dd-Hep, Gal, and GlcNAc as terminal residues.

Fraction III from OS-25218 was reduced with sodium borohydride and further fractionated by high-performance anion-exchange chromatography on CarboPac PA1 at superhigh pH to give reduced oligosaccharides 1c and 1d. Their complete structures, as well as that of oligosaccharide 1a contained in fraction II from OS-37218, were established by one- and two-dimensional 1H and 13C NMR spectroscopy using methodology described previously (15, 30) (for 1H NMR spectra and signal assignment see Supporting Information). In addition, the 1H NMR spectrum of a mixture of oligosaccharides 1a and 1b (fraction II from OS-25218) was compared with the spectra of compounds 1a, 1c, and 1d. As a result, it was found that oligosaccharides 1a and 1c differ from the related oligosaccharides 1b and 1d by replacement of a terminal d-α-D-Hep with a terminal β-Gal. Oligosaccharides 1a and 1c and oligosaccharides 1b and 1d differ from each other by the presence of a terminal α-Ko residue in compounds 1c and 1d. Therefore, the NMR data demonstrated that oligosaccharides isolated from the LPS of Y. pestis KM218 have the following structures:

\[
\begin{align*}
\text{1a} & : \beta-D-Glc\beta-(1\rightarrow4)\beta-D-Glc\beta-(1\rightarrow3)l-\alpha-D-Hep\beta-(1\rightarrow3)l-\alpha-D-Hep\beta-(1\rightarrow5)Kdo \\
\text{1b} & : \beta-D-Glc\beta-(1\rightarrow4)l-\alpha-D-Hep\beta-(1\rightarrow3)l-\alpha-D-Hep\beta-(1\rightarrow7) \\
\text{1c} & : \beta-D-Glc\beta-(1\rightarrow4)l-\alpha-D-Hep\beta-(1\rightarrow3)l-\alpha-D-Hep\beta-(1\rightarrow5)Kdo \\
\text{1d} & : \beta-D-Glc\beta-(1\rightarrow4)l-\alpha-D-Hep\beta-(1\rightarrow3)l-\alpha-D-Hep\beta-(1\rightarrow7) \\
\end{align*}
\]

Oligosaccharides 1a and 1b were evidently derived from the LPS molecules containing the second, terminal Kdo residue, which was cleaved by mild acid hydrolysis. In contrast, when present, the terminal Ko residue was not hydrolyzed and, as a result, oligosaccharides 1c and 1d were obtained. The stability of the ketosidic linkage of Ko during methanolysis has been reported (31).

Structural analysis of the oligosaccharides from Y. pestis ssp. caucasica strain 1146. In accordance with the GLC analysis of the sugar components of OS-251146 and OS-371146 (Table 2), the ESI FT-ICR MS spectrum of these molecules (Figures 2C and 2D) showed the absence of dd-Hep from the LPS.
both (Table 3). The major compounds identified in OS-37°1146 (Figure 2C) were heptasaccharide 1b and a comparable amount of the related hexasaccharide lacking Gal (1e) as well as the corresponding GlcNAc-lacking compounds 1b' and 1e'. OS-25°1146 included oligosaccharide 1d as a minor product and the corresponding GlcNAc-lacking compound 1d' as the main product (Figure 2D). Thus, in OS-25°1146 Gal occurs as a terminal monosaccharide in all molecules but only in half the molecules in OS-37°1146. The oligosaccharide from Y. pestis 1146 synthesized at both 25 °C and 37°C are distinguished from those from the Y. pestis ssp. pestis strains by a lower content of GlcNAc. Additional minor peaks were found in the mass spectra for most of the compounds of Y. pestis 1146, which had molecular masses higher by 57 Da. This was attributed to substitution of the oligosaccharides with glicine (see below). The content of the glycine-substituted compounds was markedly higher in OS-25°1146 as compared to OS-37°1146.

<table>
<thead>
<tr>
<th>variable core components</th>
<th>KM218</th>
<th>KM260(11)</th>
<th>KIMD1</th>
<th>Y. pestis ssp. caucasica</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OS-25</td>
<td>OS-37</td>
<td>OS-25</td>
<td>OS-37</td>
</tr>
<tr>
<td>a</td>
<td>H</td>
<td>d-α-β-Hep</td>
<td>β-GlcNAc</td>
<td>1371.44</td>
</tr>
<tr>
<td>b</td>
<td>H</td>
<td>d-α-β-Hep</td>
<td>β-GlcNAc</td>
<td>1341.43</td>
</tr>
<tr>
<td>c</td>
<td>α-Ko</td>
<td>d-α-β-Hep</td>
<td>β-GlcNAc</td>
<td>1607.49</td>
</tr>
<tr>
<td>d</td>
<td>α-Ko</td>
<td>β-Gal</td>
<td>β-GlcNAc</td>
<td>1577.48</td>
</tr>
<tr>
<td>e</td>
<td>H</td>
<td>H</td>
<td>β-GlcNAc</td>
<td>1179.38</td>
</tr>
</tbody>
</table>

a, b, c, d, e, f, g

| Table 3: Molecular Mass (Da) and Occurrence of Various Core Variants I in OS-25 and OS-37 from Wild-Type-LPS Y. pestis Strains |

Given estimates are based on the ESI FT-ICR MS data. * Derived by cleavage of α-Ko during mild acid hydrolysis of the LPS. Key: ++, major; +, minor; ±, trace.

On HiTrap Q anion-exchange chromatography, OS-37°1146 and OS-25°1146 eluted in essentially the same patterns as was observed for OS-37°218 and OS-25°218 from Y. pestis ssp. pestis strain KIM218 (see Figure 3), except for a lower overall amount of fraction II in OS-25°1146 that contains Ko-lacking compounds. Fraction III from OS-25°1146 and fraction II from OS-37°1146 were studied by one- and two-dimensional NMR spectroscopy as described above. This analysis confirmed the data of the MS studies and, particularly, showed that, in addition to oligosaccharides 1b and 1d, an oligosaccharide, 1e, lacking both Gal and dd-Hep was present. Overall, the major difference found in the oligosaccharides between the isolates of Y. pestis ssp. pestis and Y. pestis ssp. caucasica was the lack in the latter of oligosaccharides terminated with dd-Hep.

Structural Analysis of the Oligosaccharides from Y. pestis EV11M. The ESI FT−ICR mass spectra of OS-37°11IM and OS-25°11IM isolated from the LPS of the mutant strain Y. pestis EV11M demonstrated a mixture of Kdo−Kdo and Ko−Kdo disaccharides in both samples. OS-37°11IM and OS-25°11IM were fractionated by gel chromatography on TSK HW-20 to give two disaccharides from each preparation. The H and 13C NMR chemical shifts (see Supporting Information) and two-dimensional NMR spectroscopy data demonstrated that these are α-Kdo-(2→4)-Kdo and α-Ko-(2→4)-Kdo disaccharides, and that Kdo at the reducing end is present in a 2,7-anhydro form (see Supporting Information). The α-Ko-(2→4)-Kdo disaccharide has been isolated earlier from the LPS of Burkholderia cepacia (31) and the α-Ko-(2→4)-Kdo disaccharide from the LPS of a Re mutant of Salmonella enterica (32).

Remarkably, as opposed to the oligosaccharides in the other Y. pestis ssp. pestis strains studied, the terminal Kdo residue in OS-25°11IM and OS-37°11IM was not cleaved upon mild acid degradation of the LPS, evidently due to the lack of the heptose substituent at position 5, which, when present, renders the ketosidic linkage acid-labile.

Structural Studies of Lipid A. Fatty acid composition and the overall structure of lipid A (Figure 4) from the Y. pestis strains cultivated at different temperatures were determined using GLC−MS analysis of the acetylated methyl esters and ESI FT−ICR MS of lipid A samples isolated by mild acid degradation of the LPS. In LA-37°218 only 3-hydroxy-nystic acid [14:0(3-OH)] was present. LA-25°218 contained a major amount of 14:0(3-OH) and minor amounts of palmitoleic acid (16:1) and lauric acid (12:0). This finding is consistent with published data on Y. pestis lipid A composition (22, 23). Palmitoleic acid in Y. pestis has been formerly identified as 16:1ω9cis (33). Both LA-37°11IM and LA-25°11IM from the mutant strain Y. pestis EV11M similarly contained mainly 14:0(3-OH) but were distinguished by the presence of a minor amount of palmitic acid (16:0).

The negative ion ESI FT−ICR mass spectrum of LA-37°218 comprised two major peaks for compounds with molecular masses 1404.85 and 1178.66 Da, which corresponded to tetraacyl and triacyl lipid A species consisting of a bisphosphorylated glucosamine disaccharide backbone with four and three primary 14:0(3-OH) acyl groups attached. Furthermore, peaks for compounds carrying an additional Ara4N residue (+131 Da) were detected with lower intensity. LA-25°218 contained the same major tetraacyl and minor triacyl species and, in addition, minor pentaacyl and hexaacyl species. Each
species gave a series of ions for compounds with no, one and two Ara4N residues. As compared with the tetraacyl species, the pentaacyl species included an additional 12:0 acyl group (mass difference 182 Da) and the hexaacyl species two additional fatty acids, 12:0 and 16:1 (mass difference 182 + 236 = 418 Da), all being evidently attached as secondary acyl groups. A hexaacyl species with the same composition and, most likely, the same structure (Figure 4) has been reported as the major component in lipid A from *Y. pestis* EV40 grown at 28 °C (22).

**Structural Studies of the Whole LPS from *Y. pestis* KM218, KM260(11), and KIMD1.** The data derived from the study of the isolated oligosaccharide and lipid A components of the LPS from the *Y. pestis* ssp. *pestis* strains was used to determine the structure of the intact LPS molecule using negative ion ESI FT−ICR mass spectroscopy. In LPS-37218 the largest peak was from compound 3a (Figure 5A) with molecular mass 3240.51 Da, which corresponded to an LPS species having tetraacyl lipid A with two Ara4N residues and the Dd-Hep- and Kdo-containing oligosaccharide, which corresponded to oligosaccharide 1a but with an additional terminal Kdo residue. No peaks for higher acylated species were present. Two other major peaks corresponded to compounds lacking one and two Ara4N residues. There were also ions corresponding to the minor structures 3b and 3d (Figure 5A) wherein Gal substituted for Dd-Hep and Ko substituted for Kdo. Another minor series of ions identified in the mass spectra of LPS-37218 were those from compounds with molecular masses higher by 57 Da, which was attributed to Gly containing species. The identity of glycine was confirmed by amino acid analysis after full acid hydrolysis of the LPS. A capillary skimmer dissociation (CSD) experiment with the whole LPS revealed a pair of Y,B-fragment ions corresponding to the lipid A and core moieties, respectively, confirming that glycine is a component of the core (data not shown). The exact location of glycine in the core was not determined.

As one major immune mechanism possessed by invertebrates and mammals alike is production of anti-microbial peptides, we further investigated the effect of growth of *Y. pestis* in the presence of polymyxin B on the LPS structure. Cultivation of strain KM218 at 37 °C in the presence of polymyxin B resulted in a significant increase in the content of Ara4N and to some extent of glycine (compare the relative intensities of the mass peaks for Ara4N- and glycine-containing ions in Figures 5B and 5A), suggesting a role for these substituents in resistance to antimicrobial peptides.

Growing *Y. pestis* KM218 at 25 °C resulted in more LPS molecules with increased acylation. In addition to the tetraacyl LPS species that predominated in LPS-37218, the LPS-25218 included pentaacyl and hexaacyl species (Figure 5C). The three different acylation patterns were associated with all possible oligosaccharide glycoforms to give compounds from 3a through 3d, the Gal- & Kdo-containing compound 3b being minor (Figure 5C). Interestingly, while the secondary fatty acid 12:0 is distributed almost uniformly between the molecules with different oligosaccharide glycoforms, 16:1 is preferentially associated with Ko-containing glycoforms 1c and 1d. Only small amounts of Ara4N-lacking compounds were present, and hence, in LPS-25218 the content of Ara4N is close to stoichiometric.

No peaks corresponding to LPS with a triacyl lipid A moiety were observed in the mass spectra of the whole LPS-25218 and LPS-37218, thus indicating that those observed in the isolated lipid A (see above) were artifacts due to partial O-deacylation in the course of mild acid degradation of the LPS. The ESI MS data of the whole LPS confirmed that Kdo-lacking oligosaccharides 1a and 1b are not present in the intact LPS, likely occurring in the oligosaccharides as a result of cleavage of the terminal Kdo residue during mild acid hydrolysis.

LPS-37 and LPS-25 from *Y. pestis* KM260(11) and KIMD1 showed essentially the same MS pattern with respect to intact LPS structure except that when grown at 37 °C, *Y. pestis* KIMD1 produced a minor amount of pentaacyl LPS.
species rather than solely a tetraacyl LPS species (Table 4).

**Structural Studies of the Whole LPS from Y. pestis ssp. caucasica 1146.** Again, ESI FT-ICR mass spectrometry was used to study the structure of the intact LPS of *Y. pestis* 1146. LPS-37 1146 showed only tetraacyl lipid A species containing two Ara4N residues (Figure 5D). The major peaks were for compound 3b containing the oligosaccharide glycoform 1b, and compound 3e with the Gal-lacking glycoform 1e, as well as the corresponding compounds 3b′ and 3e′ lacking GlcNAc and some molecules that were substituted with Gly. LPS-25 1146 was distinguished by the presence of additional pentaacyl lipid A species, again with most of the molecules containing two Ara4N residues (Figure 5E). The major compounds in LPS-25 1146 were 3d with the Gal- & Ko-containing core glycoform 1d and the corresponding compound 3d′ containing oligosaccharide 1d′ lacking GlcNAc.

**Figure 5:** Charge deconvoluted negative ion ESI FT-ICR mass spectra of LPS-37 218 (A), LPS-37 218 - PMB (B), LPS-25 218 (C), LPS-37 1146 (D), LPS-25 1146 (E) and LPS-37 11M (F) and explanation of the LPS structural variants 3. LPS<sub>tetra</sub> stands for tetraacyl species etc.
The content of Gly containing species was markedly higher in LPS-25,11M as compared to LPS-37,11M.

Structural Studies of the whole LPS from Y. pestis ssp. pestis mutant strain EV11M. The ESI FT–ICR mass spectra of LPS-37,11M (Figure 5F) and LPS-25,11M were similar to each other. The major species detected were the tetraacyl LPS species with two Ara4N residues attached to either Kdo–Kdo or Ko–Kdo disaccharide core having molecular masses 2107.09 and 2123.09 Da (Figure 5, compounds 4a and 4b, respectively). Ara4N is present in nonstoichiometric amounts in both LPS preparations and its content is higher in LPS-37,11M. The mass spectra confirmed the occurrence of the fatty acid 16:0, which is evidently attached as a secondary acyl group at an undetermined position to form a minor pentaacyl lipid A species in both LPS-37,11M and LPS-25,11M. Overall, the data obtained show that ESI FT–ICR MS of the whole LPS is a useful tool for analysis of the structural diversity in the LPS of Y. pestis.

DISCUSSION

The LPS of most Gram-negative bacteria plays multiple important roles in pathogenesis, epidemiology and disease control. The structures of the LPS components, such as the O side chain, the core oligosaccharide linked to the lipid A backbone, and the lipid A itself, all affect the organism’s virulence. In addition, these structures can determine recognition of a pathogen by the innate and acquired host immune system, identification by serologic classification, and even can be a target for protective immunity. Thus, basic knowledge of the overall structure of the LPS of pathogenic Gram-negative bacteria can provide significant insight into numerous aspects of virulence, particularly when structures of related strains with greater and lesser virulence can be compared.

In the case of Y. pestis, the majority of the studies carried out on this organism have focused on the genetically homogeneous strains of bv. orientalis primarily found in North and South America, whose pauciclonality can be attributed to the lack of plague in the Americas prior to the introduction of Y. pestis by infected rats carried to the port of San Francisco from Hong Kong in 1902. The introduced strain has apparently spread throughout the Americas, where it is now found essentially in sylvatic foci, and there does not appear to have been much genetic change in American isolates over the last 100 years (4). Yet in European Russia and in Asia there is a great diversity of Y. pestis strains, many of which can be classified into different subspecies based on a variety of characteristics (4). The potential for virulence in humans of these non-American strains, and their potential as agents of bioterrorism, has to be considered because such strains could be used to avoid detection by reagents with specificity to structures such as LPS oligosaccharides and lipid A, and could also avoid immunologic control elicited by active and passive vaccination (4). In addition, because Y. pestis is introduced into mammals by fleas with generally lower, exothermically determined temperatures, structural variations in key surface molecules that are dependent on growth temperature could also impact the detection and control efforts for plague. Thus, full knowledge of the variation in attributes of these non-American Y. pestis strains, including structural variation in the LPS, is essential for proper preparedness in identifying and reacting to human infection with Y. pestis.

The data obtained here regarding the variation in the LPS structure in strains of Y. pestis found in Eurasia and Madagascar and a mutant strain previously known to have uncharacterized changes in its LPS clearly demonstrate that in wild-type-LPS strains of Y. pestis the oligosaccharide structure is affected by the growth temperature. At 37 °C, the glycoform that is mainly expressed by Y. pestis ssp. pestis has terminal Kdo and DD–Hep residues, whereas at 25 °C, four glycoforms that adopt all possible combinations of four terminal glycosyl groups (Kdo/Ko and DD–Hep/Gal) are present. Therefore, there are two types of variations in the core oligosaccharide structure: in one variation, a terminal DD–Hep residue interchanges with a Gal residue, and in another variation, a terminal Kdo residue is variably substituted with a Ko residue.

Among Yersinia species, the Gal-containing glycoforms are unique to Y. pestis, whereas a similar DD–Hep-containing glycoform occurs in Y. enterocolitica (34) and replacement of Kdo with Ko has been reported in the core of some other bacteria, e.g. in Burkholderia cenocepacia (31). The LPS of Y. pestis ssp. caucasica differed somewhat from Y. pestis ssp. pestis by the absence of DD–Hep in any of the oligosaccharide glycoforms, but, as in the other wild-type-LPS Y. pestis strains studied, the content of galactose in wild-type-LPS strains of Y. pestis is known to have reduced virulence for guinea pigs (4) and is not a significant cause of human infection, it may be that the differences in its LPS structure limit the
virulence to a restricted range of mammalian hosts, mainly voles and mice. Other studies shed further light on the genetic basis for changing the LPS structure of \emph{Y. pestis}. For example, a \textit{phoP} mutant of \emph{Y. pestis} strain GB grown at 28 °C contains dO-deoxyarabinose in lipid A, but lacks Gal, indicating that synthesis of parts of the LPS oligosaccharide are regulated by the PhoP/PhoQ two-component signal transduction system (11). Therefore, mutation in the \textit{phoP} gene (11) caused the same alteration in the core structure as did elevation of growth temperature, as demonstrated in this work. This finding suggests that when the temperature decreases, the PhoP/PhoQ system directs the LPS biosynthesis toward the Gal-containing glycoforms.

In \emph{Y. pestis} ssp. \emph{pestis}, 10–35% of the oligosaccharides can lack the terminal GlcNAc residue, and \emph{Y. pestis} ssp. \emph{caucasica} is distinguished by even a lower content of GlcNAc in the LPS. However, it is not clear if GlcNAc is a true component of the \emph{Y. pestis} core oligosaccharide or is related to the fact that the \emph{Y. pestis} LPS lacks O-antigen, and the GlcNAc residue represents what would be the first, and thus acceptor, sugar for the missing O antigen. This conclusion is consistent with the finding of the attachment of a single β-GlcNAC residue to the LPS core in an O-antigen-deficient mutant of \emph{Y. enterocolitica} 08 (34) although there was no GlcNAc in the LPS of a wild-type-LPS strain of \emph{Y. enterocolitica} 09 (35). \emph{Y. pestis} does not completely lack the O-antigen gene cluster but has a faulty one closely related to the \emph{Yersinia pseudotuberculosis} serotype O1b gene cluster (13, 36), from which \emph{Y. pestis} has likely evolved (14, 37). Comparison of the nucleotide sequences in the O-antigen gene clusters of \emph{Y. pseudotuberculosis} O1b and \emph{Y. pestis} showed almost 100% identity between all genes, except for the \textit{wzx} genes for an O-unit transporter (flipase) present in the \textit{wzy}-dependent O-antigen pathway (38), which were only 90.4% identical (14). It has been shown that flipase in \emph{E. coli} can incorporate GlcNAC onto the LPS core (39). The flipase in \emph{Y. pestis} may thus be able to translocate undecaprenyl diphasphate-linked GlcNAC onto the LPS core oligosaccharide in the absence of an O-antigen unit.

The lipid A structure of \emph{Y. pestis} also significantly varies depending on growth temperature. Although tetraacyl lipid A species are most abundant at both temperatures studied, at 25 °C additional pentaacyl and hexaacyl species are produced. Furthermore, in \emph{Y. pestis} ssp. \emph{pestis} the degree of glycosylation of phosphate groups in lipid A with Ara4N is significantly higher and is nearly stoichiometric at 25 °C compared with that at 37 °C. A similar temperature dependence of the lipid A structure has been reported for some other \emph{Y. pestis} strains (23, 40), and it was found that the \emph{Y. pestis} \textit{phoP} gene is required for Ara4N modification but not for the temperature-dependent changes in acylation (40).

In addition, we found that changes in the lipid A structure could be induced not only by changes in growth temperature but also by including polymyxin B in the growth medium, which caused a significant increase in the content of Ara4N in \emph{Y. pestis} ssp. \emph{pestis} KM218 cultivated at 37 °C. These structural variations may have important implications for the pathogenesis of \emph{Y. pestis} infection. The introduction of \emph{Y. pestis} from cooler fleas into mammalian skin is followed by formation of the inflammatory swelling of the regional lymph nodes (i.e., bubonic plague), which is likely dependent on the host response to the lipid A via interactions with toll-like receptors on macrophage-like cells in the skin and lymph nodes. Changes in lipid A following growth at mammalian temperatures could also be important in the pathogenesis of the transmissible form of pneumonic plague. Knowledge of the structural variation provided here will be key to understanding different host responses to \emph{Y. pestis} LPS that vary with growth temperature of the organism and may underlie the different disease manifestations that occur with \emph{Y. pestis} infection.

In \emph{Y. pestis} ssp. \emph{caucasica} the growth temperature had no significant influence on the substitution of lipid A with Ara4N, which was nearly stoichiometric in all samples analyzed. A structural component of the LPS of this strain was an amino acid, glycine, present on some of the oligosaccharide molecules at a currently undetermined location.

In the deep-rough mutant \emph{Y. pestis} EV11M, the LPS oligosaccharide is a disaccharide linked to a tetraacyl or pentaacyl lipid A species. The latter has a secondary fatty acid (16:0) that is different from those in wild-type-LPS strains (12:0 and 16:1). The mutation(s) in strain EV11M resulted not only in the loss of a large part of the LPS oligosaccharide and the change in fatty acid composition but also affected the system that determines the degree of lipid A acylation, which is essentially the same at both temperatures studied, although the content of Ara4N is higher at 37 °C rather than at 25 °C. We do not know if the changes in lipid A acylation in the EV11M mutant strain are secondary to the changes in the core oligosaccharide or represent independent genetic events, but the ability of \emph{Y. pestis} to be viable with the determined structural variation in the lipid A could also represent a means to manipulate these strains and alter their virulence.

There are numerous ways in which the temperature-dependent and intraspecific variations in the \emph{Y. pestis} LPS structure could have a biological significance. The production at 37 °C of a lower acylated LPS likely results in a less immunostimulatory LPS, which may compromise the host’s ability to rapidly respond with a regulated and proper inflammatory response to infection (23, 40). Such a strategy could possibly promote the virulence and person-to-person spread of pneumonic plague. The content of 4-amino-4-deoxyarabinose in lipid A, which is higher at 25 °C and increases when bacteria are grown in the presence of polymyxin B, correlates with polymyxin B resistance of \emph{Y. pestis} (ref. 23 and authors’ unpublished data). This example could indicate an adaptive response of \emph{Y. pestis} to growth in fleas, which elaborate anti-microbial peptides as a significant component of their innate immune systems. The latter finding is in agreement with a high sensitivity of a Gal-lacking \textit{phoP} mutant of \emph{Y. pestis} to polymyxin B when grown at 28 °C (11).

Overall, the findings of significant temperature-dependent variations in the LPS structure of the Eurasian/African strains of \emph{Y. pestis} representing two different subspecies is likely a key feature of enzootic and epizootic pathogenesis of this organism. Furthermore, with the bioterrorism threat presented by this organism, understanding the natural diversity in structure and function of pathogenic factors like LPS, particularly among strains not found in the Americas, will
be essential for comprehensive preparedness for dealing with this threat.

ACKNOWLEDGMENT

We thank Dr. I. A. Dunaitsev (Obolensk, Russia) for large scale production of the Y. pestis biomass, Dr. V. V. Amel’chenko (Obolensk, Russia) for drying of the biomasses and the LPS, and Mrs. A. N. Kondakova (Moscow, Russia) for assistance with ESI FT-ICR MS.

SUPPORTING INFORMATION AVAILABLE

Figures showing part of an electron impact mass spectrum of the KO–Kdo disaccharide derivative and figures showing structures and 1H NMR spectra and tables of 1H and 13C NMR data of core oligosaccharides from the lipopolysaccharide of Yersinia pestis ssp. pestis KM218. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES


BI048430F