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Functional Characterization and Biological Significance of *Yersinia pestis* Lipopolysaccharide Biosynthesis Genes

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Abstract—*In silico* analysis of available bacterial genomes revealed the phylogenetic proximity levels of enzymes responsible for biosynthesis of lipopolysaccharide (LPS) of *Yersinia pestis*, the cause of plague, to homologous proteins of closely related *Yersinia* spp. and some other bacteria (*Serratia proteamaculans*, *Erwinia carotovora*, *Burkholderia dolosa*, *Photorhabdus luminescens* and others). Isogenic *Y. pestis* mutants with single or double mutations in 14 genes of LPS biosynthetic pathways were constructed by site-directed mutagenesis on the base of the virulent strain 231 and its attenuated derivative. Using high-resolution electrospray ionization mass spectrometry, the full LPS structures were elucidated in each mutant, and the sequence of monosaccharide transfers in the assembly of the LPS core was inferred. Truncation of the core decreased significantly the resistance of bacteria to normal human serum and polymyxin B, the latter probably as a result of a less efficient incorporation of 4-amino-4-deoxyarabinose into lipid A. Impairing of LPS biosynthesis resulted also in reduction of LPS-dependent enzymatic activities of plasminogen activator and elevation of LD_{50} and average survival time in mice and guinea pigs infected with experimental plague. Unraveling correlations between biological properties of bacteria and particular LPS structures may help a better understanding of pathogenesis of plague and implication of appropriate genes as potential molecular targets for treatment of plague.

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Key words: *Yersinia pestis*, lipopolysaccharide biosynthesis, virulence, serum resistance, antimicrobial-peptide resistance, fibrinolytic activity, coagulase activity

The Gram-negative bacterium *Yersinia pestis* is the etiological agent of flea-transmitted fulminant systemic rodent zoonosis and the reason of the three devastating pandemics of plague [1, 2]. Even now up to 2000 cases of human plague are reported annually in endemic regions [3]. During the last decade bacterial factors responsible for extraordinary high virulence of *Y. pestis* [4] were extensively studied aiming at better understanding molecular mechanisms of the disease and search for novel potential molecular targets suitable for improving laboratory diagnostics, vaccine prophylaxis, and treatment of plague [5].

Lipopolysaccharide (LPS, endotoxin) is an important factor of pathogenicity of Gram-negative bacteria. The full LPS molecule (S-form LPS) consists of three well-defined domains: i) lipid A composed of sugars, fatty acids, and phosphate; it represents the endotoxic principle of the LPS and anchors it in the outer membrane; ii) a core oligosaccharide containing charged groups; and iii) an O-specific polysaccharide (O-antigen), which carries

Abbreviations: Ara4N, 4-amino-4-deoxy-L-arabinose; BHI, brain heart infusion; cfu, colony forming unit; DD-HepIV and Hep, D-*glycero*- and L-*glycero*-D-*manno*-heptose, respectively; EtNP, phosphoethanolamine; ESI FT-ICR MS, electrospray ionization Fourier transform ion-cyclotron resonance mass spectrometry; i.p., intraperitoneal; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; Ko, D-*glycero*-D-*talo*-oct-2-ulosonic acid; LPS, lipopolysaccharide; MIC, minimum inhibitory concentration; NHS, normal human serum; ORF, open reading frame; s.c., subcutaneous; 12:0, lauroyl; 16:1, palmitoleoyl (hexadec-9-enoyl); 3HO14:0, 3-hydroxymyristoyl.

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antigenic determinants eliciting specific antibody response in animals and human.

Complete-genome sequencing data have contributed significantly to understanding LPS biosynthetic mechanisms in different Gram-negative bacteria and revealed the significance of horizontal gene transfer in generating the observed structural diversity of LPS [6]. Usually genes for biosynthesis of the three structural domains of LPS are mapped in different parts of the chromosome, often as separate clusters. The majority of the LPS genes encoding enzymes of sugar and lipid metabolism fall into three major groups: i) genes involved with nucleotide sugar and fatty acid biosynthesis; ii) genes encoding transferases; and iii) processing genes [7, 8].

Yersinia pestis is a clone evolved from the enteric pathogen *Yersinia pseudotuberculosis* 1500-20,000 years ago [9]. In the course of divergence *Y. pestis* lost as a result of mutations many genes useful for enteropathogenic yersiniae, *Y. pseudotuberculosis* and *Y. enterocolitica*, but unnecessary for the vector-borne lifestyle of *Y. pestis* [10]. Particularly, it lost the ability to synthesize O-antigen [11], and, as a result, the LPS of *Y. pestis* is restricted to the core and lipid A moieties (R-form LPS).

The O-antigen-deficient LPS promotes resistance of *Y. pestis* to serum-mediated lysis [12-15], which is necessary for survival and growth of the bacteria in mammalian blood and for transmission between mammals by insects [1, 15-18]. It determines also bacterial resistance to cationic antimicrobial peptides [13, 14, 19], a key component of the innate immunity in both mammals and insects [20]. Reduction in the degree of acylation of lipid A of *Y. pestis* at the mammalian host temperature (37°C) [21-25] makes this pathogen-associated molecule invisible for pattern recognition Toll-like receptor 4 [26] and enables uncontrolled multiplication of *Y. pestis* in the host resulting in septic shock and death necessary for further transmission of the pathogen by infected fleas searching for a new host [16, 27].

Recently, the fine LPS structures have been reported in representatives of various intraspecies groups of *Y. pestis* grown at 37, 21-28, or 6°C to simulate the conditions of their life in the organism of warm-blooded hosts, poikilothermal insects, and animals during winter hibernation, respectively [21-24, 28-34]. Variations in the LPS structure of *Y. pestis* have been documented and found to depend on the growth temperature, composition of the nutrient media, and belonging of *Y. pestis* strains to a certain intraspecies group. However, genes responsible for *Y. pestis* LPS biosynthesis are characterized scarcely [25, 26, 35-38]. Elucidation of their roles in bacterial virulence would implicate genes as molecular targets for treatment of plague.

This work aims at unraveling the impact of particular LPS components on *Y. pestis* pathogenicity, including resistance of the bacteria to various antimicrobial factors, and to understand better the biological significance of the temperature-dependent LPS structural variations. For these purposes, we identified genes that are involved with biosynthesis of the *Y. pestis* LPS, generated the corresponding nonpolar mutants, and studied LPS structures and virulence-related biological properties of the mutant strains.

Parts of this work were presented at the 9th and 10th International Symposia on *Yersinia* (October 10-14, 2006, Lexington, Kentucky, USA [14] and October 23-27, 2010, Recife, Brazil) and 46th Oholo Conference "The Challenge of Highly Pathogenic Microorganisms – Mechanism of Virulence and Novel Medical Countermeasures" (October 25-29, 2009, Eilat, Israel [15]).

MATERIALS AND METHODS

Bacterial strains. *Yersinia pestis* virulent strain 231 and its attenuated derivative KM260(11) were obtained from the Russian Research Anti-Plague Institute "Microbe" (Saratov, Russia). Characteristics of the strains are given in Table 1. All experiments with strain 231 and its virulent derivatives were performed using BSL3 facilities. *Escherichia coli* strain S17-1 λ*pir* was used for construction of recombinant plasmids and as a donor for conjugative transfer of these plasmids [39]. Bacterial cultures were started from glycerol stocks (20% glycerol, –20°C).

Growth of bacteria. For mutagenesis and animal challenge experiments, *Y. pestis* strains were grown at 25°C for 48 h on brain heart infusion (BHI; HiMedia Laboratories Pvt. Limited, India) supplemented with 2% agar, pH 7.2. BHI agar with 5% sucrose was used for selection of *Y. pestis* recombinants with the help of the suicide vector pCVD442 [40]. *Escherichia coli* strain S17-1 λ*pir* and its derivatives were grown at 37°C for 24 h in Luria–Bertani broth, pH 7.2 [41], or on the same medium supplemented with 2% agar. For LPS isolation, *Y. pestis* strains were grown at 25 or 6°C as described [23].

Animals. BALB/c mice (~20 g each) (Lab Animals Breeding Center, Russian Academy of Sciences, Pushchino, Moscow Region, Russia) and guinea pigs (~260 g each) (Lab Animals Breeding Center, Russian Academy of Medical Sciences, Stolbovaya, Moscow Region, Russia) of both sexes were used in animal experiments that were approved by the Bioethics Committee of the State Research Center for Applied Microbiology and Biotechnology. Animals were kept in cages in groups at the most of five and three, respectively, and allowed to feed and drink *ad libitum* during experiment terms.

Animal passage. Purification of populations of *Y. pestis* strains from clones that might reduce their virulence during storage in glycerol stocks or after gene-engineering manipulations was performed by animal passage in mice [42].

Table 1. Bacterial strains and plasmids

Y. pestis strain and plasmid	Description	Reference
231	pFra$^+$, pCD$^+$, pPst$^+$; bv. antiqua subsp. *pestis**; wild-type virulent strain	[18]
231Δ*wabD* (ΔYPO0187::*nptII*)	Δ*wabD*; derived from strain 231; Km^R	
231Δ*waaL* (ΔYPO0417::*nptII*)	Δ*waaL*; derived from strain 231; Km^R	
231Δ*waaQ* (ΔYPO0416::*nptII*)	Δ*waaQ*; derived from strain 231; Km^R	
231Δ*waaE* (ΔYPO0054::*nptII*)	Δ*waaE*; derived from strain 231; Km^R	
231Δ*hldE* (ΔYPO0654::*nptII*)	Δ*hldE*; derived from strain 231; Km^R	
KM260(11)	pFra$^+$, pCD$^-$, pPst$^-$; derived from strain 231; avirulent	[23]
KM260(11)pKD46	KM260(11) harboring plasmid pKD46; Ap^R	
KM260(11)Δ*wabC* (ΔYPO0186::*nptII*)	Δ*wabC*; derived from strain KM260(11)pKD46; Km^R	
KM260(11)Δ*wabD* (ΔYPO0187::*nptII*)	Δ*wabD*; derived from strain KM260(11)pKD46; Km^R	
KM260(11)Δ*wecA* (ΔYPO3866::*nptII*)	Δ*wecA*; derived from strain KM260(11)pKD46; Km^R	
KM260(11)Δ*waaL* (ΔYPO0417::*nptII*)	Δ*waaL*; derived from strain KM260(11)pKD46; Km^R	
KM260(11)Δ*wabC/waaL* (ΔYPO0186::*nptII*/YPO0417::*cat*)	Δ*wabC/waaL*; derived from strain KM260(11)pKD46; Km^R Cm^R	
KM260(11)Δ*wabD/waaL* (ΔYPO0187::*nptII*/YPO0417::*cat*)	Δ*wabD/waaL*; derived from strain KM260(11)pKD46; Km^R Cm^R	
KM260(11)Δ*waaQ* (ΔYPO0416::*nptII*)	Δ*waaQ*; derived from strain KM260(11)pKD46; Km^R	
KM260(11)Δ*waaE* (ΔYPO0054::*nptII*)	Δ*waaE*; derived from strain KM260(11)pKD46; Km^R	
KM260(11)Δ*waaF* (YPO0057::*nptII*)	Δ*waaF*; derived from strain KM260(11)pKD46; Km^R	
KM260(11)Δ*waaC* (YPO0056::*nptII*)	Δ*waaC*; derived from strain KM260(11)pKD46; Km^R	
KM260(11)Δ*hldE* (YPO0654::*nptII*)	Δ*hldE*; derived from strain KM260(11)pKD46; Km^R	
KM260(11)Δ*waaA* (YPO0055::*nptII*)	Δ*waaA*; derived from strain KM260(11)pKD46; Km^R	
KM260(11)Δ*arnT* (YPO2421::*nptII*)	Δ*arnT*; derived from strain KM260(11)pKD46; Km^R	
KM260(11)Δ*lpxM* (YPO2063::*nptII*)	Δ*lpxM*; derived from strain KM260(11)pKD46; Km^R	
KM260(11)Δ*lpxP* (YPO3632::*cat*)	Δ*lpxP*; derived from strain KM260(11)pKD46; Cm^R	
KM260(11)Δ*eptB* (YPO4013::*nptII*)	Δ*eptB*; derived from strain KM260(11)pKD46; Km^R	
EV line NIIEG	pFra$^+$, pCD$^+$, pPst$^+$, Δ*pgm*; Russian vaccine strain; bv. *orientalis* subsp. *pestis**; avirulent	[18]
E. coli S17-1 λ*pir* B	λ*pir* lysogen of S17-1 (*thi pro hsdR*$^-$ *hsd*M$^+$ *recA* RP4 2-Tc::Mu-Km::Tn*7* (Tp^R Sm^R Pm^S)	[39]

Note: All *Y. pestis* mutants were generated in this work.
* For details of biovar-subspecies interrelations see review [18].

***In silico* search for the LPS biosynthesis genes and bioinformatic analysis of sequence data.** Search for LPS associated genes was performed through annotated genome sequences of *Y. pestis* strains (http://www.ericbrc.org/portal/eric/yersiniapestis?id=enteropathogens&subid=yersiniapestis). When relevant information was absent, genes with already revealed functions from other bacteria were used as templates for search of orthologs in genome sequence of *Y. pestis* strain CO92 [43] by using the BLAST network service (National Center for Biotechnology Information).

The nomenclature proposed by P. R. Reeves et al. [44] is used for designation of bacterial polysaccharide biosynthesis genes.

General DNA manipulations were performed essentially as described [45]. DNA restriction endonucleases, T4 DNA ligase, *E. coli* DNA polymerase (Klenow fragment), and alkaline phosphatase were used as recommended by the suppliers.

Mutant construction. The chromosomal in-frame deletion mutants in attenuated strains were constructed by allelic exchange using a kanamycin (neomycin resistance gene, *nptII*) or chloramphenicol (*cat*) resistance cassette following PCR synthesis of their sequences with short flanking homologous regions of the *Y. pestis* target DNA [46] (see Table 1 in Supplement to this paper on the journal website at http://www.protein.bio.msu.ru/biokhimiya). The resistance cassettes were obtained by PCR amplification using plasmids pUTKm and pKD3 for *nptII* and *cat*, respectively, as template (Table 1). The PCR products were introduced by electroporation into *Y. pestis* KM260(11)pKD46 as described [47]. Recombinant colonies were selected on kanamycin or chloramphenicol BHI agar plates. Correct insertion of the antibiotic resistance cassette was verified by PCR.

The chromosomal in-frame deletions in LPS biosynthesis genes of *Y. pestis* virulent strain 231 were carried out using suicide vector pCVD442 [40]. Mutant alleles of Δ*wabD*, Δ*waaL*, Δ*waaQ*, Δ*waaE* and Δ*hldE* were amplified from chromosomal DNA of *Y. pestis* strains KM260(11)Δ*wabD*, KM260(11)Δ*waaL*, KM260(11)Δ*waaQ*, KM260(11)Δ*waaE*, and KM260(11)Δ*hldE*, respectively, using primer pairs YPO0187f/YPO0187r for Δ*wabD*, YPO0417f/YPO0417r for Δ*waaL*, YPO0416f/YPO0416r for Δ*waaQ*, YPO0054f/YPO0054r for Δ*waaE* and YPO0654f/YPO0654r for Δ*hldE* (see Table 1 in Supplement to this paper on the journal website at http://www.protein.bio.msu.ru/biokhimiya). Fragments containing the affected genes were ligated into the SmaI site of suicide vector pCVD442 [40]. The ligated plasmids were electroporated into *E. coli* S17-1 λ*pir* cells to form plasmids pCVD442Δ*wabD*::*nptII*, pCVD442Δ*waaL*::*nptII*, pCVD442Δ*waaQ*::*nptII*, pCVD442Δ*waaE*::*nptII*, and pCVD442Δ*hldE*::*nptII*. Then the plasmids constructed were introduced into the wild-type *Y. pestis* 231 recipient from *E. coli* S17-1 λ*pir* by conjugation using polymyxin B for contra-selection to produce $Km^RAp^RPm^R$ or $Km^RCm^RAp^RPm^R$ merodiploid transconjugants. The selected transconjugants were plated onto BHI agar with 10% sucrose and grown at 28°C for 2 days. The resultant $Suc^RKm^RAp^S$ or $Suc^RKm^RCm^RAp^S$ colonies were screened for accuracy of mutations using PCR with the corresponding primer pair. Colonies of Km^RAp^S or $Km^RCm^RAp^S$ *Y. pestis* double-crossover mutants (Table 1) for each suicide plasmid were collected together, and 10^6 cfu of each culture were used for intraperitoneal (i.p.) challenge of mice (four animals for each strain). The cultures on antibiotic-free BHI agar isolated from spleens of animals that succumbed to infection were used for second i.p. challenge of mice with 10^4 cfu dose (four animals for each strain). The strains isolated from the second round of animal passage were used for further studies. Correct insertion of the antibiotic resistance cassette was verified by PCR.

PCR verification of accuracy of mutations. Three PCR variants were used to show that all mutated DNA fragments have the expected structure. The common test primers for gene *nptII* included k1 and k2, and those for *cat* included c1 and c2 (see Table 1 in Supplement to this paper on the journal website at http://www.protein.bio.msu.ru/biokhimiya). Two reactions were performed using flanking locus-specific primers with the respective common test primer (k1, k2, c1, or c2) to test for new junction fragments. A third reaction was carried out with the flanking locus-specific primers to verify the loss of the parental (non-mutant) fragment and the presence of the new mutant-specific fragment. Control wild-type colonies were tested side-by-side.

Isolation of LPS and SDS-PAGE. LPS was extracted from dried cells of each strain with phenol/chloroform/light petroleum ether [48] and purified by repeated ultracentrifugation (105,000*g*, 4 h) following enzymatic digestion of nucleic acids and proteins [49]. The purity of the isolated LPS preparations was evident from the lack of protein and nucleic acid contaminants.

SDS-glycine PAGE with silver staining of the gels was performed as described [50].

Mass spectrometry. Electrospray ionization Fourier transform ion-cyclotron resonance mass spectrometry (ESI FT-ICR MS) was performed using an Apex II instrument (Bruker Daltonics, USA) equipped with a 7 T actively shielded magnet and an Apollo electrospray ion source as previously described [23]. Negative ion mass spectra were acquired and processed using standard experimental sequences as provided by the manufacturer. Mass accuracy has been checked through external calibration. Samples (~10 ng/μl) were dissolved in a 50 : 50 : 0.001 (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 drying gas temperature to 150°C. To help assignment of mass peaks [34], capillary skimmer dissociation was induced by increasing the capillary exit voltage from −100 to −350 V. The spectra, which showed several

charge states for each component, were charge deconvoluted, and mass numbers given refer to the monoisotopic masses of the neutral molecules.

Resistance to bactericidal action of polymyxin B. The sensitivity of *Y. pestis* strains grown at 25°C to cationic antimicrobial peptides was tested using different doses of polymyxin B to calculate the minimum inhibitory concentration (MIC) as described previously [13].

Resistance to bactericidal action of human serum. A pool of normal human serum (NHS) was obtained from ten non-immunized healthy volunteers. The complement was inactivated by incubation of NHS at 56°C for 30 min. Bactericidal properties of NHS were tested by incubation of bacteria grown at 25°C with serum for 1 h as described [13].

Coagulase and fibrinolytic activities were determined as described [51]. A positive coagulase test (++) was represented by a solid clot; a positive test (+) by any degree of incomplete clotting (from a loose clot to a solid clot in liquid plasma); a negative test (–) by the absence of clotting. A positive fibrinolysis test (++) was represented by complete clot lysis; a positive test (+) by any degree of lysis; a negative test (–) by a solid clot.

Virulence of *Y. pestis* strains for mice and guinea pigs. Six naïve animal groups (each of 40 mice of BALB/c line) and five naïve animal groups (each of 12 guinea pigs) were administered subcutaneously (s.c.) in the right inguinal region with serial 10-fold dilutions (10^7 to 10^0 cfu, five mice for one dose and 10^4 to 10^1 cfu, three guinea pigs for one dose) of two-day *Y. pestis* agar cultures grown at 25°C. Humane endpoints were strictly observed. Animals that succumbed to infection were sacrificed and examined bacteriologically. The remaining animals were observed for 21 days and then humanely killed by carbonic gas inhalation. Mortality times were calculated, and the LD_{50} and 95% confidence intervals (CI) were determined according to the method of Kärber [52].

RESULTS

***In silico* search for the *Y. pestis* LPS biosynthesis genes.** Search through annotated genome sequences of *Y. pestis* strains for LPS genes resulted in selection of several candidate genes for mutagenesis (see Table 2 in Supplement to this paper on the journal website at http://www.protein.bio.msu.ru/biokhimiya). Taking into account that the majority of mutations affecting early steps of the lipid A assembly are lethal for bacteria [53] and that the corresponding enzymes are conserved within different enterobacteria [6], attention was given to genes for synthesis of the core and late steps of lipid A synthesis. In the *Y. pestis* CO92 genome, we found three gene loci numbered 1-3 predicted to be involved with LPS core biosynthesis (Fig. 1a).

Locus 1 includes five ORFs flanked by genes encoding phosphopantetheine adenylyltransferase (CoaD) and 2-amino-3-ketobutyrate coenzyme A ligase (Kbl). ORF YPO0058 was identified as the gene for ADP-D-*glycero*-β-D-*manno*-heptose 6-epimerase based on a high level of identity. Four other ORFs (YPO0054-YPO0057) were assigned as genes for transferases responsible for the assembly of the conserved inner core region by sequential adding two 3-deoxy-D-*manno*-oct-2-ulosonic acid residues (KdoI and KdoII) to lipid A by bifunctional Kdo transferase (WaaA) followed by transfer of two L-*glycero*-D-*manno*-heptose residues (HepI and HepII) catalyzed by heptosyltransferases I and II (WaaC and WaaF, respectively) and adding glucose to HepI by glucosyltransferase (WaaE) (Fig. 1b).

From two ORFs present in locus 3, YPO0416 was suggested to encode heptosyltransferase III (WaaQ), which adds HepIII to HepII to complete the inner core assembly. The other ORF, YPO0417, was assigned as *waaL*, which encodes ligase that links the O-antigen to the core.

Locus 2 that follows a gene for taurine dioxygenase (TauD) contains two ORFs, YPO0186 and YPO0187, for putative new glycosyltransferases. These genes named *wabC* and *wabD* were found to be responsible for adding D-*glycero*-D-*manno*-heptose (DD-HepIV) or galactose, respectively, to HepIII (see below).

Putative genes for synthesis of lipid A, including those for late acylation lauroyltransferase (YPO2063, *lpxM*) and palmitoleoyltransferase (YPO3632, *lpxP*), glycosylation with 4-amino-4-deoxy-L-arabinose (Ara4N) (YPO2421, *arnT*) and phosphorylation (YPO1276, *lpxT*) as well as genes for synthesis of Hep (YPO0654, *hldE*) and GlcNAc undecaprenyl diphosphate (YPO3866, *wecA*) were found elsewhere on the chromosome (Fig. 1a).

An additional BLAST search was performed for *Y. pestis* genes homologous to sequences coding for phosphoethanolamine (PEtN) and glycine transferases, which are non-stoichiometric constituents of *Y. pestis* LPS core [23, 32]. As a result, an ortholog for *eptB* (*yhjW*) (YPO4013) coding for the PEtN transferase in *E. coli* [54] was located (Fig. 1a). No homolog for the *wbnG* gene predicted for coding glycine transferase in *Shigella dysenteriae* [55] was found, probably owing to a substantial difference between enzymes that add glycine to an amino group in *S. dysenteriae* and to a hydroxy group in *Y. pestis*.

Phylogenetic relationships of *Y. pestis* LPS biosynthetic enzymes to other bacteria. *In silico* revelation of phylogenetic connection of putative *Y. pestis* LPS-biosynthetic enzymes with homologous enzymes of closely related bacteria indicated that the identity of almost all of these proteins is 100% within *Y. pestis* species and 98-100% within the *Yersinia* genus. These data are in agreement with a high level of similarity of the core and lipid A structures in yersiniae LPS [24, 33, 34, 56, 57].

Yersinia pestis enzymes WaaE, WaaA, WaaC, WaaF, HldD, HldE, GmhB, GmhA, WecA (Rfe), EptB (YhjW), ArnT (PmrK), LpxM (MsbB), and LpxP have more than 70% amino acid identity and absolute functional identity

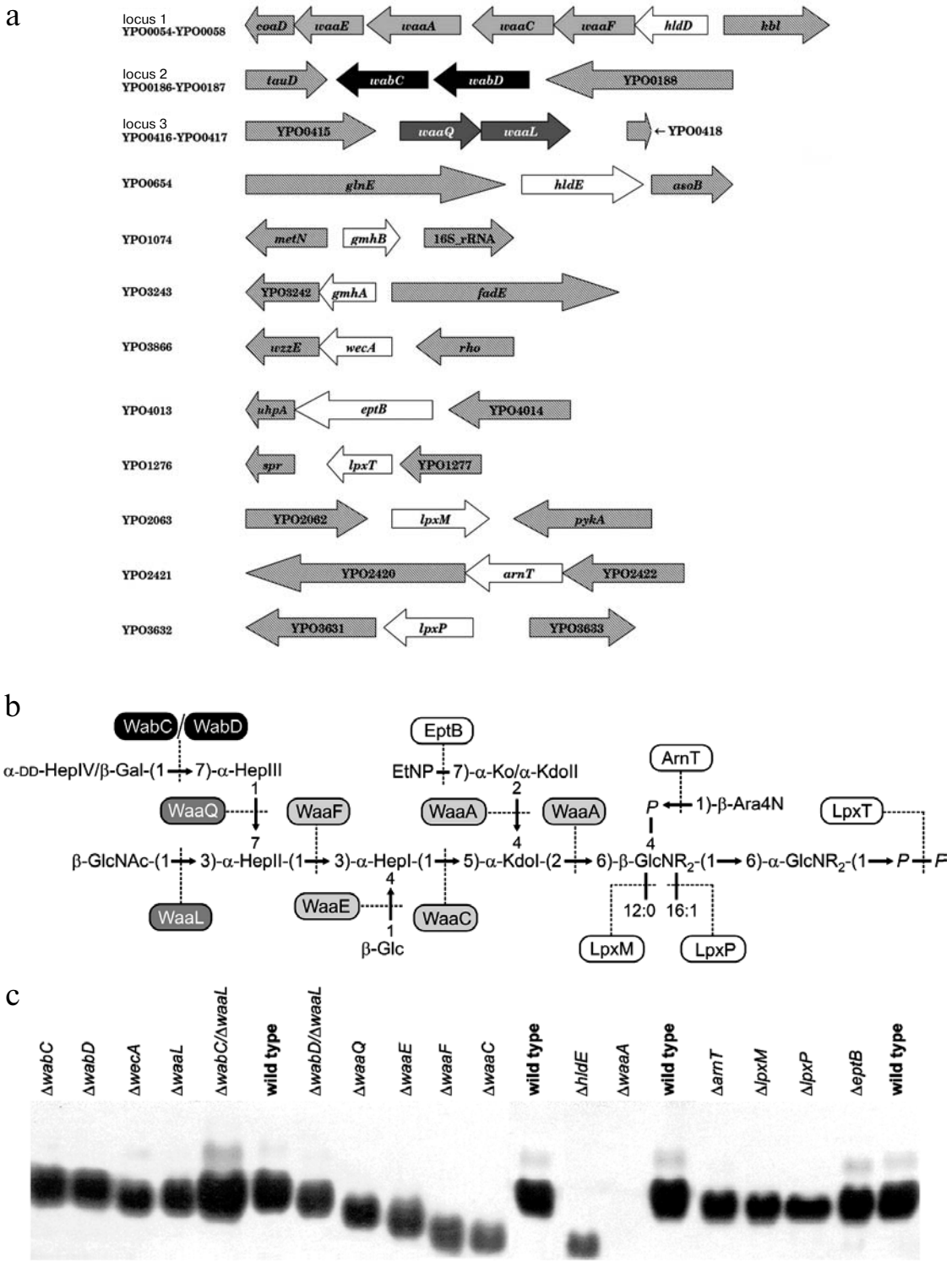


Fig. 1. a) Organization of some LPS biosynthetic genes on *Y. pestis* chromosome. The *waa* genes in loci 1 and 3 and *wab* genes in locus 2 are rendered with light gray, gray, and black, respectively. b) Structure of *Y. pestis* LPS and functional assignment of glycosyl- and acyl-transferases involved with LPS biosynthesis. Enzymes encoded by *waa* genes in loci 1 and 3 and *wab* genes in locus 2 are rendered with light gray, gray and black, respectively. R stands for 3-hydroxymyristoyl. In cultures grown at 25°C, DD-HepIV and Gal, KdoII and Ko alternate pairwise whereas at 37°C DD-HepIV and KdoII predominate. EtNP in the core is mainly expressed in cultures grown at 6°C. Diphosphate in lipid A is expressed at 37°C and replaced with Ara4NP at 25°C. Glycine linked to HepI (authors' unpublished data) is not shown. c) SDS-PAGE of LPS samples from *Y. pestis* KM260(11) and derived LPS mutants. (Fig. 1b is reproduced from [15] with kind permission from Springer Science+Business Media B.V.).

with those of distantly related bacteria, such as *Serratia proteamaculans*, *Klebsiella pneumoniae*, *Erwinia carotovora*, *Burkholderia* spp., *Photorhabdus luminescens*, *Legionella pneumophila*, *S. dysenteriae*, *E. coli* and *Salmonella enterica* (see Table 2 in Supplement to this paper at http://www.protein.bio.msu.ru/biokhimiya). However, WaaQ, WabC, and WaaL of *Y. pestis* and distantly related counterparts are less than 64% identical, and galactosyltransferase WabD is identical to glycosyltransferases of other bacteria at the most by 43%. Among the bacteria whose genomes have been analyzed, the most probable donors of *Y. pestis*/*Y. pseudotuberculosis* LPS biosynthetic genes (or recipients of similar genes from the same bacterial donor) are *S. proteamaculans* for *waa* genes in loci 1 and 3 as well as genes *hldE*, *gmhB*, *gmhA*, *wecA*, *eptB*, *lpxT*, *lpxM*, *arnT* and *lpxP*; *E. carotovora* subsp. *atroseptica* for gene *hldD* in locus 1; *Burkholderia dolosa* and *P. luminescens* subsp. *laumondii* for *wab* genes in locus 2.

Generation of LPS mutants. Each single gene with predicted functions in LPS biosynthesis and, in addition, two pairs of genes, YPO0186/YPO0417 (*wabC*/*waaL*) and YPO0187/YPO0417 (*wabD*/*waaL*) were inactivated in avirulent *Y. pestis* strain KM260(11) by using PCR products [46] with kanamycin and/or chloramphenicol resistance cassettes. Each mutant was complemented *in trans* by the corresponding wild-type gene (data not shown) to rule out the possibility that the phenotype observed is caused by a polar effect or a spontaneous mutation elsewhere in the genome. All generated mutants were shown to be non-polar.

In the wild-type virulent strain *Y. pestis* 231 similar homologous recombinations were performed using a set of plasmids derived from suicide vector pCVD442 [40] and resulted in generation of YPO0054 (*waaE*), YPO0187 (*wabD*), YPO0416 (*waaQ*), YPO0417 (*waaL*), and YPO0654 (*hldE*) mutants.

SDS-PAGE of mutant LPSs. Lipopolysaccharide was isolated from each *Y. pestis* KM260(11) mutant derivatives grown at 25°C and analyzed by SDS-PAGE (Fig. 1c). The LPSs of YPO0054 (*waaE*), YPO0056 (*waaC*), YPO0057 (*waaF*), YPO0416 (*waaQ*), and YPO0654 (*hldE*) mutants moved faster than the wild-type LPS, thus indicating that, as expected, inactivation of the corresponding genes caused deep changes in the LPS structure. The LPS from YPO0055 (*waaA*) mutant with impaired gene for Kdo transferase was not stained on the gel by silver, a characteristic of a core-lacking LPS consisting of a lipid A portion only [58]. Mutations in YPO0186 (*wabC*), YPO0187 (*wabD*), YPO0417 (*waaL*), and YPO3866 (*wecA*) genes responsible for adding outer core monosaccharides (Fig. 1b), YPO4013 (*eptB*) gene decorating the inner core with EtNP, and lipid A modifying genes YPO2063 (*lpxM*), YPO2421 (*arnT*), and YPO3632 (*lpxP*) altered LPS profiles on gels only insignificantly.

Structures of mutant LPSs and confirmation of LPS biosynthesis gene functions. The LPS of each mutant derived from *Y. pestis* KM260(11) was cleaved by mild acid hydrolysis to lipid A and core oligosaccharide; the latter was purified by gel chromatography on Sephadex G-50. The isolated core and lipid A samples and/or intact LPSs were studied by ESI FT-ICR MS in the negative ion mode. Mass spectra of the intact LPS of *arnT* mutant, core oligosaccharides from *waaL*, *wabC*, *wabD*, *waaQ*, and *waaF* mutants [14] and lipid A of *lpxM* mutant [38] have been reported earlier. The data obtained were compared with the data of wild-type LPS, whose structure has been elucidated earlier [23, 24, 28, 30-34].

waaL (YPO0417) mutant. The LPS from the *waaL* mutant [14] showed essentially the same MS pattern as the wild-type LPS except that peaks for GlcNAc-containing compounds were absent from the spectrum. As in wild type, there were present oligosaccharides having either Gal or DD-HepIV at the non-reducing terminus (Fig. 1b and Table 2) and those containing or lacking a terminal residue of D-*glycero*-D-*talo*-oct-2-ulosonic acid (Ko). The last variants were derived from LPS containing either terminal KdoII or terminal Ko residue on KdoI (Fig. 1b), the former, but not the latter, being split off upon mild acid degradation of the LPS [23]. The absence of GlcNAc from the *waaL* mutant suggested that ligase WaaL encoded by the inactivated gene was responsible for adding GlcNAc to the LPS core.

The same GlcNAc-lacking LPS chemotype was detected in the *wecA* (YPO3866) mutant, which is unable to synthesize undecaprenyl diphosphate derivative of GlcNAc, a donor substrate for WaaL.

wabC (YPO0186) and wabD (YPO0187) mutants, and wabC/waaL and wabD/waaL double mutants. Core oligosaccharides from *wabC* and *wabD* mutants were distinguished by the absence of either terminal DD-HepIV or terminal Gal (Table 2) [14], and, therefore, the encoded enzymes are heptosyltransferase IV and galactosyltransferase, respectively (Fig. 1b). As expected, the core of *wabC/waaL* and *wabD/waaL* double mutants lacks both GlcNAc and DD-HepIV or both GlcNAc and Gal, respectively (Table 2). The absence of either of these monosaccharides does not interfere with biosynthesis of the remaining part of the LPS core.

waaQ (YPO0416) mutant. The core of the *waaQ* mutant was devoid of HepIII (Table 2) [14]. As expected, no DD-HepIV and Gal were present as these monosaccharides are carried by HepIII (Fig. 1b). GlcNAc that is linked to HepII occurs only in a minority of molecules. Therefore, *waaQ* encodes heptosyltransferase III, and ligase WaaL transfers GlcNAc to the core in the absence of HepIII inefficiently. The synthesis of the rest of the inner core region was not affected by inactivation of *waaQ*.

waaE (YPO0054) mutant. The mass spectrum of the LPS from *waaE* mutant (Fig. 2a) showed the major series of peaks for compounds with the core moiety containing Ko, KdoI, HepI, and HepII (Table 2) and lipid A moiety consisting of bisphosphorylated diglucosamine backbone with four 3HO14:0 groups and one Ara4N residue (meas-

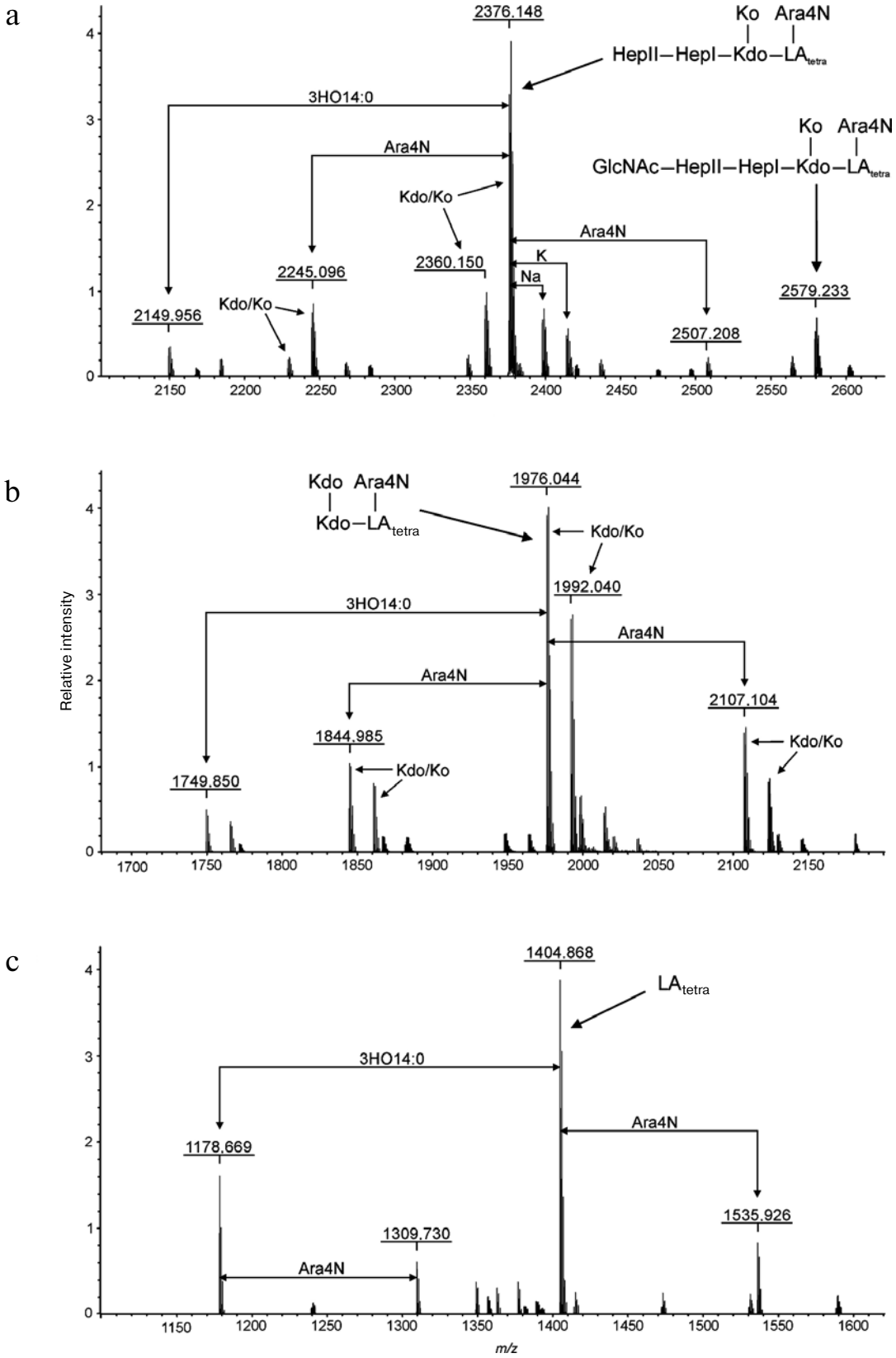


Fig. 2. Charge deconvoluted negative ion ESI FT-ICR mass spectra of the whole LPS of *waaE* (a), *waaC* (b) and *waaA* (c) mutants derived from *Y. pestis* KM260(11). Peaks with mass difference 16 Da belong to compounds with either KdoII or Ko on KdoI. LA_{tetra} stands for tetra-acyl lipid A containing four 3-hydroxymyristoyl groups (3HO14:0).

Table 2. Biological properties of wild-type strain *Y. pestis* 231 and derived LPS mutants

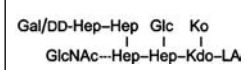
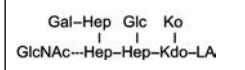
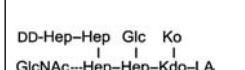
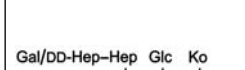
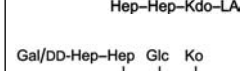
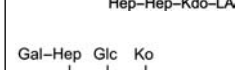
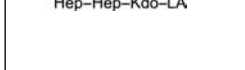
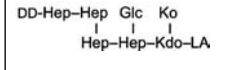
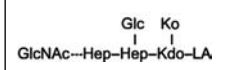
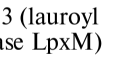
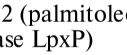
Mutation in gene (encoded enzyme)	Core oligosaccharide structure	MIC of polymyxin B for *Y. pestis* strain, U/ml	Number of live bacteria (log cfu/ml) after 1 h of incubation in		Pla activities		Virulence for			
							mice		guinea pigs	
			NHS	NHS*	coagulase	fibrinolytic	LD_{50}, cfu	mean time to death, days	LD_{50}, cfu	mean time to death, days
1	2	3	4	5	6	7	8	9	10	11
Wild type		1250/20[Ψ]	7.0 ± 0.81	7.2 ± 0.73	++	++	2 (1-4)	4.7 ± 1.23	7 (2-27)	8.5 ± 0.8
YPO0186 (HepIV-transferase WabC)		2500	6.4 ± 0.65	6.8 ± 0.43	++	++	n.d.	n.d.	n.d.	n.d.
YPO0187 (Gal-transferase WabD)		1250	6.3 ± 0.51	6.1 ± 0.72	++	++	8 (1-32)	4.2 ± 0.73	n.d.	n.d.
YPO3866 (GlcNAc-1-P-transferase WecA)		1250	6.9 ± 0.71	7.1 ± 0.83	+	+	n.d.	n.d.	n.d.	n.d.
YPO0417 (ligase WaaL)		625	6.3 ± 0.71	6.8 ± 0.62	+	+	13 (2-50)	4.5 ± 0.80	32 (8-126)	9.0 ± 1.5
YPO0186/YPO0417 (HepIV-transferase/ ligase WabC/WaaL)		625	6.4 ± 0.91	6.5 ± 0.56	+	+	n.d.	n.d.	n.d.	n.d.
YPO0187/YPO0417 (Gal-transferase/ligase WabD/WaaL)		625	6.4 ± 0.35	6.5 ± 0.74	+	+	n.d.	n.d.	n.d.	n.d.
YPO0416 (HepIII-transferase WaaQ)		**20**	**3.6 ± 0.53**	6.7 ± 0.70	+	+	5 (1-16)	4.3 ± 0.91	15 (4-58)	10.0 ± 1.1
YPO0054 (Glc-transferase WaaE)		**20**	**3.5 ± 0.24**	6.8 ± 0.46	+	+	**32 (8-126)**	**7.9 ± 0.80**	**>10^4**	**>21**
YPO0057 (HepII-transferase WaaF)		**20**	**2.3 ± 0.19**	6.1 ± 0.56	-	-	n.d.	n.d.	n.d.	n.d.

Table 2 (Contd.)

1	2	3	4	5	6	7	8	9	10	11
YPO0056 (HepI-transferase WaaC)		**10**	**2.0 ± 0.33**	6.5 ± 0.52	-	-	n.d.	n.d.	n.d.	n.d.
YPO0654 (D-β-D-Hep-7-P-kinase/ D-β-D-Hep 1-P-adenyl transferase HldE)		**10**	**2.2 ± 0.18**	6.6 ± 0.85	-	-	**$2.0 \cdot 10^5$** ($5.0 \cdot 10^4$-$7.9 \cdot 10^5$)	**11.5 ± 0.53**	**>10^4**	**>21**
YPO0055 (Kdo-transferase WaaA)		**5**	**0**	5.3 ± 0.71	-	-	n.d.	n.d.	n.d.	n.d.
YPO2421 (Ara4N-transferase ArnT)		**20**	6.5 ± 0.55	6.7 ± 0.71	++	++	n.d.	n.d.	n.d.	n.d.
YPO4013 (PEtN-transferase EptB)		1250/20^{Ψ}	6.7	6.9	+	+	n.d.	n.d.	n.d.	n.d.
YPO2063 (lauroyl transferase LpxM)		1250	6.9	7.1	++	++	n.d.	n.d.	n.d.	n.d.
YPO3632 (palmitoleoyl transferase LpxP)		1250	6.3	6.8	++	++	n.d.	n.d.	n.d.	n.d.

Note: LA, wild-type lipid A; LA*, lipid A deficient in Ara4N, lauroyl (12:0) or palmitoleoyl (16:1) groups; Ko*, core deficient in PEtN. Dotted line indicates a non-stoichiometric amount of GlcNAc. When both are present, DD-HepIV and Gal alternate at the non-reducing end. In all strains, terminal Ko is partially replaced with terminal Kdo. Significant changes in biological properties are indicated in bold face. NHS*, heat-inactivated NHS; 20^{Ψ}, MIC at 6°C; n.d., not determined. For explanation of symbols (++), (+), (–) see "Materials and Methods".

ured and calculated molecular masses 2376.15 Da). There were also minor peaks for compounds that differ in the replacement of Ko with KdoII (2361.17 Da), the absence of Ara4N (2245.10 Da) or one of the 3HO14:0 groups (2149.96 Da) and the presence of a GlcNAc residue (2579.24 Da). The lack from the *waaE* mutant LPS of both Glc and HepIII suggested that *waaE* encodes glucosyltransferase and that incorporation of HepIII into the core requires prior addition of Glc to HepI. As in the *waaQ* mutant, GlcNAc-containing core variants were present in the *waaE* mutant only in small proportions.

waaF (YPO0057) mutant. A single heptose residue (HepI) was found in the LPS of the *waaF* mutant (Table 2) [14], and, hence, the inactivated gene encoded heptosyltransferase II. The lack of Glc from the *waaF* mutant suggested that its addition to the core requires a prior transfer of HepII onto HepI.

waaC (YPO0056) mutant. Only Kdo (Ko) mono- and disaccharides were obtained by mild acid degradation of the *waaC* mutant LPS (Table 2). The mass spectrum of the whole LPS (Fig. 2b) revealed the major compounds having KdoII→KdoI or Ko→KdoI disaccharide core linked to lipid A containing four 3HO14:0 groups and one Ara4N residue (measured molecular masses 1976.05 and 1192.04 Da, calculated molecular masses 1976.03 and 1992.02 Da, respectively). Minor peaks in the spectrum belonged to the compounds containing two or no Ara4N residue (2107.11 and 2123.10 Da; 1844.99 and 1860.98 Da, respectively) and a compound with only three 3HO14:0 groups in lipid A (1749.85 Da). The lack of any heptose from the core suggested that *waaC* encodes heptosyltransferase I.

The same LPS chemotype was found in the *hldE* (YPO0654) mutant, which is unable to synthesize the activated Hep nucleotide derivative.

waaA (YPO0055) mutant. No core was found among mild acid degradation products of the LPS of the *waaA* mutant. The mass spectrum of the LPS (Fig. 2c) showed that it is restricted to the lipid A moiety with four (major) and three (minor) 3HO14:0 groups (measured molecular masses 1404.86 and 1178.67 Da, calculated molecular masses 1404.85 and 1178.66 Da, respectively). Therefore, the inactivated gene encoded Kdo transferase WaaA, a bifunctional enzyme that catalyzes transfer of both KdoI to lipid A and KdoII to KdoI [6].

Only a minority of LPS molecules in the *waaA* mutant contain an Ara4N residue (molecular masses 1535.92 and 1309.73 Da for compounds with three and four 3HO14:0 groups, respectively). A relatively low content of Ara4N (on the average not more than one residue per molecule) was observed also in lipid A of other *Y. pestis* LPS mutants with deeply truncated core, including the *waaE* and *waaC* mutants (Fig. 2, a and b). In contrast, in wild-type LPS [23] as well as in the *waaL*, *wabC*, and *wabD* mutants both phosphate groups in lipid A are almost completely glycosylated with Ara4N. This phenomenon may be accounted for by a lower efficiency of Ara4N transfer to lipid A in the LPS having an incomplete inner core region or, especially, no core as in the *waaA* mutant.

eptB (YPO4013) mutant. A core variant containing an EtNP group on terminal Ko was found to be abundant in the wild-type LPS grown at 6°C (Fig. 1b) [32]. No EtNP was present in the LPS of the *eptB* mutant cultivated at the same temperature, while the carbohydrate core backbone and lipid A in the mutant and wild type were the same. Therefore, *eptB* encoded EtNP transferase.

arnT (YPO2421) mutant. The mass spectrum of the LPS showed that the *arnT* mutant is unable to incorporate Ara4N into lipid A [14] and, therefore, the inactivated gene encoded Ara4N transferase ArnT. Inactivation of *arnT* had no influence on the LPS core structure.

lpxM (YPO2063) mutant. No hexaacyl form was found in lipid A from the *lpxM* mutant, but it had a pentaacyl form with four 3HO14:0 and one 16:1 groups. None of the lower acylated variants included a lauroyl group 12:0 either [38]. No other significant changes were observed either in lipid A or in the core of the mutant LPS. Therefore, it was concluded that *lpxM* encoded lauroyl transferase LpxM.

lpxP (YPO3632) mutant. As in the *lpxM* mutant, no hexaacyl form was found in lipid A from the *lpxP* mutant, but in this case the highest acylated variant contained four 3HO14:0 and one 12:0 groups. None of the lower acylated variant included 16:1 group either, and, therefore, *lpxP* encoded palmitoleoyl transferase LpxP.

Inactivation of any LPS biosynthesis gene but *waaA* does not alter growth rate of bacteria. Most mutants, except for the *waaA* mutant, showed practically the same growth rates as the wild type. Therefore, the inactivated genes were not essential for bacterial growth, and the mutations did not cause any adverse pleiotropic effects that could affect the growth rate.

Mutation in the *waaA* gene resulted in a significant deceleration of bacterial growth, and the final yield of *waaA* mutant biomass after fermentation was 8-10-fold lower than that of the other strains studied.

Resistance of mutants to bactericidal action of polymyxin B and normal human serum. The *waaQ*, *waaE*, *waaF*, *waaC*, *hldE*, *waaA*, and *arnT* mutants were 31 to 250 times less resistant to polymyxin B (MIC < 20 U/ml) than the parental *Y. pestis* strain (Table 2). Strains carrying mutations in the *wabC*, *wabD*, *wecA*, and *waaL* genes as well as the *wabC*/*waaL* and *wabD*/*waaL* double mutants showed essentially the same resistance as the wild type (MIC > 625 U/ml).

The *waaQ*, *waaE*, *waaF*, *waaC*, *hldE*, and *waaA* deficient strains with deeply truncated LPS core were highly susceptible to the bactericidal action of NHS (Table 2). The serum killing was complement-mediated as these mutants were resistant to heat-inactivated serum. Other mutants, including the *arnT* mutant with an Ara4N-lacking LPS, were almost as resistant to NHS as the parental strain.

The full LPS core is necessary for enzymatic activity of plasminogen activator. Fibrinolytic and plasmocoagulase activities of plasminogen activator Pla were tested in *Y. pestis* strains with mutations in the genes of the LPS core biosynthetic pathway (Table 2). It was found that both activities are first decreased (in *wecA*, *waaL*, *wabC*/*waaL*, *wabD*/*waaL*, *waaQ*, and *waaE* mutants) and then totally abolished (in *waaF*, *waaC*, *hldE*, and *waaA* mutants) with a gradual reduction of the core.

Virulence of mutants in mice and guinea pigs. A gradual truncation of the LPS core was accompanied by a decrease in bacterial virulence (Table 2). In all cases the animal death was dose-related. However, the reduction in virulence remained behind the decrease in the bacterial resistance to the innate immunity factors. For instance, the *waaQ* mutant deficient in HepIII transferase was highly susceptible to polymyxin B and NHS but was as virulent as the parental strain for both animals. In mice, further reduction of the core, e.g. as in the *waaE* mutant deficient in synthesis of glucosyltransferase, was accompanied by a slight but reliable increase in LD_{50} values (2→32 cfu) and mean times to death (4.7→7.9 days). In any case such LD_{50} is extremely low, and the *waaE* mutant is still highly virulent for mice. The least virulent for mice was the *hldE* mutant deficient in synthesis of Hep and possessing a core restricted to a Kdo→Kdo or Ko→Kdo disaccharide. In guinea pig, neither *waaE* nor *hldE* mutant caused animal death during 21 days of experiment.

Bacteriological examination of spleen from animals that succumbed to infection indicated that they were all positive for *Y. pestis* during experiment terms. Indeed, a heavy growth of bacteria was observed on plates inoculated with homogenized spleen specimens. Organs from survivors infected with strain 231 and the derived *wabD*, *waaL*, *waaQ* (mice and guinea pigs), or *waaE* (mice) mutants on day 22 postinfection were all negative for *Y. pestis*. However, 60 to 90% of spleen specimens from survivors of both animals challenged with *hldE* mutant and guinea pigs infected with the *waaE* mutant on day 22 postinfection were positive for *Y. pestis*: solitary colonies of bacteria were observed on plates inoculated with homogenized spleen specimens.

DISCUSSION

Bioinformatic analysis of the fully sequenced genome of *Y. pestis* combined with mutagenesis showed that genes for the LPS core biosynthesis are distributed between several regions on the chromosome (Fig. 1a). The functions of these genes were confirmed by the LPS structure elucidation of the corresponding mutants. The *waa* genes for synthesis of the inner core region, which are rather conserved within enteric bacteria, are clustered in two loci.

A minimal inner core is necessary for normal growth of bacteria. Indeed, although viable like the *waaA* mutant of *E. coli* [58], the *waaA* mutant of *Y. pestis* fully devoid of the core was significantly impaired in growth rate. One of the *waa* loci includes *waaL*, a gene known to encode ligase WaaL that ligates the O-antigen to the core. However, *Y. pestis* is devoid of the O-antigen, and WaaL functions as a transferase that adds a single GlcNAc residue to the core [23].

Two *wab* genes in the third locus are responsible for adding on the alternative basis of galactose or DD-HepIV to the core. Expression of the galactosyl transferase gene *wabD* is under the control of the two-component PhoPQ regulatory system [29], but the biological significance of this regulation remains obscure. Some *Y. pestis* strains belonging to biovar microtus non-*pestis* subspecies *altaica*, *caucasica*, and *xilingolensis* [18, 59] lack DD-HepIV in the LPS core [13, 24, 34]. In *Y. pestis* subsp. *caucasica* Pestoides F and *Y. pestis* subsp. *xilingolensis* 91001 that also belong to non-*pestis* subspecies (bv. microtus), the gene *wabC* for DD-HepIV transferase is inactivated by a mutation resulting in inability to synthesize the full 326-amino acid wild-type enzyme but probably only its non-functional derivatives of 192 or 34 amino acids. A similar mutation can be expected in DD-HepIV-lacking strains with the elucidated LPS core structure as they are affiliated within the same or closely phylogenetically related subspecies/genotypes [59].

Yersinia pestis LPS biosynthetic genes show a high-level identity of to analogous proteins of bacteria from different phylogenetic groups (see Table 2 in Supplement to this paper at http://www.protein.bio.msu.ru/biokhimiya). This finding combined with dispersed location of the genes throughout *Y. pestis* chromosome (Fig. 1a) suggests their multistage horizontal gene transfer into the genome of the *Yersinia* progenitor. On the other hand, *wabC* mutations in representatives of subspecies *altaica*, *caucasica*, and *xilingolensis* indicate an ongoing process of reductive intraspecies microevolution in the course of adaptation of *Y. pestis* to circulation in populations of specific rodent hosts (*Microtus* spp.).

In *Y. pestis*, one of the most successful strategies to evade the immune response in the host is realized by temperature-dependent modifications of lipid A [21-25]. Reduced acylation of lipid A at the mammal host temperature [21-25] makes the bacterium unrecognized by pattern recognition Toll-like receptor 4 [26]. As a result, the innate immunity system becomes unable to respond properly, and the bacteria can multiply unopposed by the normal host defense mechanisms. LPS is also responsible for overcoming the other innate immunity branches such as cationic antimicrobial peptides [13, 14, 19] and serum-mediated lysis [12-14].

Resistance of *Y. pestis* to cationic antimicrobial peptides depends on the content of the cationic monosaccharide Ara4N [22], whose temperature-dependent

incorporation to lipid A is regulated by the PhoPQ regulatory system [22, 37]. As expected, the *arnT* mutant of *Y. pestis* subsp. *pestis* 260(11) deficient in Ara4N was highly susceptible to polymyxin B (this work), as reported for *arnT* mutants of some other bacteria [60]. A failure to locate the gene coding for glycine transferase precluded testing a possible contribution of glycine, another cationic component of *Y. pestis* LPS, to the resistance to cationic antimicrobial peptides as hypothesized by us earlier [13].

Single and double mutants of *Y. pestis* subsp. *pestis* with impaired genes responsible for adding the lateral core monosaccharides (*wabC*, *wabD*, *waaL*) were as resistant to polymyxin B as the parental strain. Surprisingly, *Y. pestis* subsp. *caucasica* 1146, having essentially the same LPS chemotype as *wabC* mutant of strain 260(11), was highly sensitive to polymyxin B [13, 23]. This finding suggests that the inability to incorporate DD-HepIV into the LPS in strain 1146 is no reason of the susceptibility to polymyxin B. Further studies are necessary to unravel the mechanisms of the susceptibility of non-*pestis* strains to cationic antimicrobial peptides.

Serum resistance is necessary for survival and multiplication of the cause of plague in mammal blood for further transmission of bacteria by insects to new hosts [1, 16, 17, 27]. *Yersinia pestis* R-LPS incorporated into liposomes was shown to ensure serum resistance, in contrast to S-LPS from serum-susceptible yersiniae phenotypes [12]. On the other hand, serum resistance in *Y. pestis* is mediated by an outer membrane protein Ail (OmpX) [61, 62], and changes in the LPS structure may influence the Ail conformation and specific activity [62]. Our data of *Y. pestis* LPS mutants show that the full inner core composed of six sugar residues (KdoI, KdoII or Ko, HepI, HepII, HepIII and Glc) is required for the resistance to NHS, whereas the outer core monosaccharides (DD-HepIV, Gal and GlcNAc) are of little importance.

It can be expected that *ail* is not functional in *Y. pestis* subsp. *caucasica* strain 1146 [13] and other serum-susceptible non-*pestis* strains. However, this gene could be amplified in PCR from 1146 DNA and its product seen in silver-stained SDS-PAGE of outer membrane proteins [62] from strain 1146 at the same level as similar proteins from serum-resistant strains (authors' unpublished data). Sequencing of *ail* from strain 1146 (GenBank accession number FJ447341) showed that it contains three additional nucleotides responsible for inclusion of additional Ser148 into the Ail amino acid sequence. The same insertion was reported for another subspecies *caucasica* strain Pestoides F (GenBank accession number NC_009381). According to the reported model of the Ail topology in the outer membrane [63], Ser148 is inserted into the sequence of the third cell surface loop. This loop is believed to be involved with serum resistance of bacteria and may be the main reason of the serum susceptibility of subspecies *caucasica* strains.

Plasminogen activator Pla, a transmembrane protease belonging to omptins, is one of the recognized *Y. pestis* pathogenicity factors responsible for systemic spread of bacteria in the host organism [16, 64]. In some *Y. pestis* strains, Pla expression is obligatory for full subcutaneous virulence. In contrast, in all isolates of subspecies *caucasica*, which are naturally deficient in plasmid pPst (pPla, pPCP1, or pYP) coding for Pla, or some artificial Pla^- mutants of subspecies *pestis* strains, the absence of Pla does not affect the high subcutaneous virulence, which is comparable to that of classical wild-type Pla^+ strains (for review see [18]). Recently, it has been demonstrated that the presence of an S-type LPS with a long-chain O-antigen inhibits the action of *Y. pestis* Pla, whereas R-LPS is necessary for proper folding of Pla and manifestation of its enzymatic activities [65, 66]. It has been found also that temperature-induced modifications to the LPS of *Y. pestis* affect plasminogen activation by Pla [67].

Our data on Pla fibrinolytic and plasmocoagulase activities in *Y. pestis* strains with gradual truncation of the core suggest that all eight wild-type *Y. pestis* core constituent sugars are necessary for maximal enzymatic activities of Pla. A LPS with a core consisting of seven to five sugar residues was still able to ensure the fibrinolytic and coagulase activities, though at noticeably reduced levels, whereas a deeper truncation resulted in inactivation of Pla.

The *wabD*, *waaL*, and *waaQ* mutants of *Y. pestis* subsp. *pestis* were still extraordinarily virulent in both mice and guinea pigs (Table 2). Strains belonging to biovar microtus non-*pestis* subspecies *altaica*, *caucasica*, and *xilingolensis* [18, 59] unable to incorporate DD-HepIV into the core [24, 34] are virulent only for mice [18, 59]. Therefore, the LPS deficiency in the terminal monosaccharide is no reason of selective virulence of biovar microtus strains. Only a reduction of the core down to five sugar residues caused a noticeable decrease in subcutaneous virulence of *Y. pestis* subsp. *pestis* for guinea pigs ($\geq 10^3$ cfu). A further truncation of the core to two sugar residues abolished virulence for both mice and guinea pigs.

In summary, *Y. pestis* knock-out mutants with two or less sugar residues in the LPS core were not only highly susceptible to antimicrobial cationic peptides and NHS but also avirulent in murine and guinea pig infection models. This finding demonstrated that the LPS structure is critical for plague pathogenesis, and *waaC*, *hldE*, and *waaA* or their protein products can be considered as promising candidates for targeting *Y. pestis* virulence using specific inhibitors [68]. As the identities of the corresponding enzymes to non-yersiniae protein homologs are ≤89%, it seems possible to fit an inhibitor to each of the targets that will not affect normal commensal microflora in the mammalian host.

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