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Characterization of a Transposon Tn*5*-Generated Mutant of *Yersinia pestis* Defective in Lipooligosaccharide Biosynthesis

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Abbreviations: Ara4N, 4-amino-4-deoxy-L-arabinose; CAMP, cationic antimicrobial peptides; CFU, colony-forming unit; ESI ICR FT, high resolution ion cyclotron resonance mass spectrometry with Fourier transformation and electrospray ionization; Glc, glucose; GlcNAc, *N*-acetylglucosamine; Hep, L-*glycero*-L-*manno*-heptose; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; Ko, D-*glycero*-D-*talo*-oct-2-ulosonic acid; LPS, lipopolysaccharides; MIC, minimum inhibitory concentration; NHS, normal human serum; PMB, polymyxin B; HIS, heat-inactivated serum (incubated at 56°C for 30 min to inactivate complement).

Abstract—To identify *Yersinia pestis* genes involved in the microbe's resistance to cationic antimicrobial peptides, the strategy of random transposon mutagenesis with a Tn*5* minitransposon was used, and the library was screened for detecting polymyxin B (PMB) susceptible mutants. The mutation responsible for PMB-sensitive phenotype and the lipopolysaccharide (LPS) structure were characterized for the *Y. pestis* strain KM218-A3. In this strain the mini-Tn*5* was located in an open reading frame with the product homologous to the *E. coli* protein GmhB (82% identity) functioning as D-*glycero*-D-*manno*-heptose-1,7-diphosphate phosphatase. ESI FT ICR mass spectrometry of anions was used to study the structure of the unmodified LPS of *Y. pestis* KM218-A3, and molecules were revealed with the full-size LPS core or with two types of an incomplete core: consisting of Kdo-Kdo or Ko-Kdo disaccharides and Hep-(Kdo)-Kdo or Hep-(Ko)-Kdo trisaccharides. The performed complementation confirmed that the defect in the biological properties of the mutant strain was caused by inactivation of the *gmhB* gene. These findings indicated that the *gmhB* gene product of *Y. pestis* is essential for production of wild-type LPS resistant to antimicrobial peptides and serum.

Keywords: *Yersinia pestis*, lipopolysaccharide, heptose biosynthesis, serum resistance, antibiotic resistance

The pathogenicity of *Y. pestis* in the body of a susceptible host can be realized if the causative agent of plague possesses pathogenic factors with different functional direction that would protect it against the innate and acquired immunity of the macroorganism. The more ancient evolutionary innate immunity system acts immediately after the inculcation of a pathogen and activates phagocytosis and inflammatory reactions. In the inflammatory reactions a great role belongs to the complement system, cytokines, lysozyme, properdin, leucine, and β-lysine, as well as to other antimicrobial agents including cationic peptides. During the evolution of interrelationships of the micro- and macroorganism, resistance mechanisms to the innate immunity factors were formed in bacterial pathogens, in particular, in pathogenic yersiniae that allow them to survive and multiply in the body of a susceptible host [1-3]. The resistance to cationic antimicrobial peptides (CAMP) found in many tissues of mammals and insects [4-6] is an important factor of *Y. pestis* pathogenicity [7, 8]. This publication considers determination of new genes whose products allow *Y. pestis* to escape the bactericidal action of cationic peptide antibiotics. To obtain *Y. pestis* strains susceptible to polymyxin B as a representative of such antibiotics, we have chosen a strategy of random transposon mutagenesis with the minitransposon Tn*5*.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this work were obtained from the State Collection of Pathogenic Microorganisms and Cell Cultures (SCPM-Obolensk). Their characteristics are presented in Table 1.

Growing the bacteria. The *Y. pestis* strain for mutagenesis was grown at temperature 25°C for 48 h on a solid nutrient medium Brain heart infusion (HiMedia, India) [14] supplemented with hemolyzed blood to 2% (v/v) at pH 7.2. The *Escherichia coli* strain S17-1 λ*pir* and its derivatives were grown at temperature 37°C for 24 h in Luria–Bertani medium [15] at pH 7.2. For isolating lipopolysaccharides (LPS), the *Y. pestis* strains were grown at temperature 25°C as described earlier [16].

Insertion mutagenesis. Insertion mutants were obtained using a conjugation method [17]. As a recipient, we used the *Y. pestis* strain KM218 resistant to polymyxin B and as a donor we used the *E. coli* strain S17 λ*pir* containing the plasmid pUTKm [11]. *Yersinia pestis* conjugants were counter-selected on media containing gentian

violet (1 : 100,000) (w/v) (DIAEM, Russia) supplemented with kanamycin (40 μg/ml) or ampicillin (100 μg/ml).

Gene engineering. Chromosomal DNA of bacteria and DNA of plasmids were isolated as recommended by the producer, using a Genomic DNA Purification Kit and a DNA Extraction Kit (Fermentas, Lithuania). The restriction and ligation were performed using standard methods [15]. The *E. coli* cells were transformed by electroporation using the method described in the Operating Manual to the Electroporator 2510 device (Eppendorf, Germany).

DNA sequencing was performed by Syntol (Russia). The resulting nucleotide sequences were analyzed using the BLAST2 program on the NCBI server.

Complementation. For complementation, a low-copy expressing vector pAE-gfp was constructed. To do this, the gene *gfp*/*asv* including the SD-sequence from the plasmid pKK214GFP/ASV [18] was cloned in the pBluescript II SK(–) vector by the restriction sites PstI and EcoRI. The SmaI-SalI fragment of the resulting plasmid pBlu-gfp was cloned into the plasmid pACYC184 by the sites EcoRV-SalI. On constructing the plasmid for complementation, the gene *gfp*/*asv* from pAE-gfp [13] was cut by the restriction sites NdeI-SalI and replaced by the coding sequence of the *gmhB* gene amplified with the primers GmhBNdeI (5′-TTGATGCATATGGTGA-CTCAGTCCGTT-3′) and GmhBSalI (5′-CTTGTCGACCTATTTATAACGCGC-3′) (Syntol).

Isolation of LPS and SDS-PAGE. LPS was isolated from dry bacterial cells with a mixture of phenol, chloroform, and petroleum ether [19] and purified by sequential enzymatic degradation of nucleic acids and proteins and repeated ultracentrifugation (105,000*g*, 4 h) [16].

For screening of polymyxin-sensitive clones, LPS was isolated by the method of Hitchcock and Brown [20]. SDS-PAGE was performed as described earlier [21]. To visualize LPS, the gel was stained with ammonia silver oxide solution after oxidation with periodic acid as recommended by Tsai and Frash [22].

Mass spectrometry. The structures of the LPS components were established using high resolution ion cyclotron resonance mass spectrometry with Fourier transformation and electrospray ionization (ESI ICR FT). The mass spectra were taken with recording anions using an ApexII device (Bruker Daltonics, USA) as described earlier [16].

Minimal inhibitory concentration (MIC) of polymyxin B was determined

using the routine approach of dilution in liquid nutrient medium [23].

Study on *Y. pestis* interactions with serum. Bactericidal properties of serum were determined using a slight modification of the method of Barnes et al. [24]. Viability of the studied strains after cultivation in normal human serum with intact complement (NHS) or with heat-inactivated complement (HIS) was determined after planting onto dense nutrient medium.

RESULTS

Obtaining mini-Tn*5* mutants sensitive to polymyxin B (PMB). The genes responsible for the resistance of the plague microbe to PMB were determined using random transposon mutagenesis. This method allowed us to obtain a large number of mutant clones due to inserting of the minitransposon Tn*5* encoding kanamycin resistance into genome of the recipient strain. As a recipient, we used a plasmid-free derivative of the *Y. pestis* vaccine strain EV line NIIEG, KM218, that was resistant to the bactericidal action of PMB and human serum. The structure of LPS from this strain was determined by us earlier [16].

In total, in five separate experiments with mutagenesis we obtained more than 3,000 clones of *Y. pestis* KM218 with the minitransposon Tn*5* inserted into a chromosome, and among these clones eight isolates were detected with the phenotype Pol^S. These mutants were denominated as KM218-A3, KM218-7, KM218-7K, KM218-45K, KM218-457K, KM218-804K, KM218-2048K, and KM218-3209K. The MIC of PMB for the sensitive mutants was 20-250 times lower than the MIC for the *Y. pestis* parent strain KM218 (Table 2). Two of the eight clones (KM218-457K and KM218-A3) were sensitive also to the bactericidal activity of the normal human serum complement. After incubation for 1 h in NHS, the number of colony-forming units (CFU) was significantly lower than after incubation in serum containing the heat-inactivated complement. The *Y. pseudotuberculosis* strain EV11M with the core part of the LPS completely lacking heptose residues [16] displayed a high sensitivity to PMB and NHS.

The analysis by SDS-PAGE revealed that the migration rates of LPS of PMB-sensitive isolates were not different from the migration rate of the parent strain *Y. pestis* KM218. The migration rate of LPS of the mutant strain KM218-457K was higher (data not presented). LPS from the mutant strain KM218-A3 formed in the polyacrylamide gel two bands, one of which had the same migration rate as LPS of the initial strain

KM218 (Fig. 1) and the other was on the level of LPS isolated from the strain *Y. pseudotuberculosis* EV11M whose core was shown by us to completely lack heptose residues [16]. The structure of LPS of *Y. pestis* KM218-A3 and the mutation responsible for this phenotype were characterized later.

Determination of the location of minitransposon Tn*5* in strain KM218-A3. The genomic DNA isolated from mutant KM218-A3 was hydrolyzed with restrictases BamHI and BglII and ligated with plasmid pUC19 hydrolyzed by the site BamHI. The resulting ligase mixture was used for transformation of *E. coli* DH5α competent cells, and clones resistant to ampicillin (the plasmid marker) and kanamycin (the mini-Tn*5* marker) were selected. Plasmids isolated from the resulting clones included insertions of ~1800 and 5000 bp that presented the DNA sequence of *Y. pestis* flanking the mini-Tn*5* insertion and 900 bp DNA of mini-Tn*5*. To determine the nucleotide sequence of the plague microbe in the region of the inserted transposon, both chains of the DNA insertion were sequenced. The resulting nucleotide sequences were compared to the DataBank NCBI (https://www.ncbi) using the BLAST2 program. It was established that in the strain *Y. pestis* KM218-A3 the mini-Tn*5* was located at the beginning of the open reading frame *YPO1074* (NC_003143.1, 1216470-1217036). The product of this gene was 82% identical with protein GmhB of *E. coli* (WP_001608862.1) that functions as D-*glycero*-D-*manno*-heptose-1,7-diphosphate phosphatase [25].

Complementation of the Tn*5*-mutation in the *Y. pestis* strain KM218-A3. To confirm that the obtained defect of biological properties of the mutant strain was caused by inactivation of the corresponding gene, an intact gene *gmhB* was cloned in the constructed expressing vector pACYC-gfp. The resulting plasmid pAE-*gmhB* was inserted by electroporation into the *Y. pestis* strain KM218-A3. The electrophoretic profile of the LPS preparation isolated from the complemented strain *Y. pestis* KM218-A3/pAE-*gmhB* was similar to the initial strain *Y. pestis* KM218, and this confirmed that the cloned gene *gmhB* successfully complemented the created mutation (Fig. 1).

Structural characteristic of LPS from *Y. pestis* strain KM218-A3. The ESI ICR FT mass spectrum of the unmodified LPS obtained from the KM218-A3 strain grown at 25°C (Fig. 2) contained the major peak at *m/z* 3227,4670 corresponding to the lipid A tetracyl form with two 4-amino-4-deoxy-L-arabinose (Ara4N) residues and the 1d type core with the stoichiometric content of *N*-acetylglucosamine (GlcNAc) (Fig. 2). GlcNAc was identified by us earlier in the LPS of the "wild" strain KM218 [16]. The minor components corresponded to compounds with the 1a-1c type cores differed from 1d by replacement of D-*glycero*-D-*talo*-oct-2-ulosonic acid (Ko) by 3-deoxy-D-*manno*-

oct-2-ulosonic acid (Kdo) and/or of Gal by D-*glycero*-D-*manno*-heptose (DD-Hep) (Table 3). Moreover, they contained one or two additional C12:0 and C16:1 fatty acid residues, respectively (Fig. 2). A small number of molecules included one Ara4N residue in the lipid A.

The ESI ICR FT mass spectrum revealed molecules with an incomplete core of two types. One of them was represented by Kdo-Kdo (2a, major form) and Ko-Kdo (2c, minor component) disaccharides, and the other – by Hep-(Kdo)-Kdo (3a, minor component) and Hep-(Ko)-Kdo (3c, major form) trisaccharides (Fig. 2).

Sensitivity of *Y. pestis* strain KM218-A3 to polymyxin B. On everyday successive replanting of the studied strain KM218-A3, the MIC of polymyxin B increased (31.25-125 μg/ml), not reaching the MIC value of the parental strain (Fig. 3). However, despite the replanting, the strain remained sensitive to the bactericidal action of the NHS complement.

DISCUSSION

The ability of *Y. pestis* for intracellular reproduction and its resistance to the bactericidal action of cationic antimicrobial peptides and human serum complement play an important role in the pathogenesis of plague [7, 26-30]. It is established that the *Y. pestis* resistance to bactericidal action of cationic peptides is higher than the resistance of *Y. pseudotuberculosis* and *Y. enterocolitica.* The increase in the resistance correlated with the increase in the reduction degree of LPS. The complete absence in the *Y. pestis* LPS structure of negatively charged O-polysaccharide chains resulted in the minimum ability of the bacteria to sorb cationic peptides [31]. Our data have confirmed that the strain *Y. pestis* subsp. *pestis* KM218 is highly resistant to PMB at temperature 25°C [7]. In the present study, we have obtained PMB-sensitive mutants of the strain *Y. pestis* KM218 and detected a genetic locus involved in providing the resistance to this antimicrobial agent.

It was shown earlier by us, as well as by other researchers, that mutations damaging the structure of oligosaccharide core or of lipid A in representatives of the *Yersinia* genus led to decrease in the resistance of the strains to PMB, β-defensins, cathelicidins, and cecropins [32-39]. It has been shown that in several bacteria, the region of LPS inner core is a bacteriophage receptor necessary to withstand the damaging action of human serum component and for resistance against opsonization

and capture by neutrophils [39-42]. In the present study, we have established that in the PMB-sensitive strain *Y. pestis* KM218-A3 mini-Tn*5* is located in the beginning of the open reading frame *YPO1074* (NC_003143.1, 1216470-1217036). The product of this gene was necessary also for resistance of the *Y. pestis* strain to the human serum complement.

The function of the gene *YPO1074* of *Y. pestis* was assumed based on the homology determined by BLAST search. The product of the open reading frame *YPO1074* of *Y. pestis* shares 82% identity to GmhB (D-*glycero*-D-*manno*-heptose-1,7-diphosphate phosphatase) of *E. coli* (earlier *YaeD*), which converts D-*glycero*-D-*manno*-heptose 1,7-diphosphate into D-*glycero*-D-*manno*-heptose 1-phosphate. This reaction is a stage of biosynthesis of ADP-L-*glycero*-D-*manno*-heptose, which is a substrate for WaaC and WaaF heptosyltransferases in *E. coli* and other gram-negative microorganisms [25]. The pathway of ADP-L-*glycero*-D-*manno*-heptose biosynthesis and the genes participating in this pathway were described in detail earlier [25, 43]. Thus, the gene *YPO1074* was identified as *gmhB* and named respectively.

According to the findings, LPS of *Y. pestis* strain KM218 with mutation in the *gmhB* gene contained several type molecules of the oligosaccharide core: the full-size molecules characteristic for the initial strain KM218 and molecules with the core reduced to disaccharide (Kdo-Kdo or Ko-Kdo) and trisaccharide (Hep-(Kdo)-Kdo or Hep-(Ko)-Kdo). The core represented by disaccharides of Kdo and Ko was detected by us earlier on the determination of structure of LPS of the highly sensitive to PMB laboratory strain *Y. pseudotuberculosis* EV11M [16].

Kneidinger et al. [25] showed that the deletion in the *gmhB* gene in *E. coli* strain K-12 led to disorder in the LPS core biosynthesis manifesting in appearance of molecules with heptose residues and without them. They supposed that these different molecules could indicate a partial compensation of the affected synthesis of ADP-L-*glycero*-D-*manno*-heptose and found that such compensation was due to the bifunctional protein HisB of *E. coli*, which also displayed histidinol phosphate phosphatase activity. In the case of the *Y. pestis* mutant KM218-A3, the different degree reduction of the LPS core molecules and the detected insignificant growth of the PMB-resistance in the process of replanting seemed to be also associated with compensatory mechanisms. However, in spite of this, the strain retained the sensitivity to PMB, not reaching the MIC value of the highly resistant parental strain. Moreover, the strain KM218-A3 retained the sensitivity to NHS, not depending on the replanting.

Thus, this study on strain *Y. pestis* KM218-A3 obtained by random transposon

mutagenesis with the minitransposon Tn*5* has shown that the change of the *YPO1074* gene whose product is involved in a stage of ADP-L-*glycero*-D-*manno*-heptose synthesis affects synthesis of the pentasaccharide fragment of LPS. This leads to appearance of LPS molecules with shortened core and to sensitivity of the plague microbe to the bactericidal activity of normal human serum and polymyxin B. We showed earlier that knockout mutants of *Y. pestis* with two or fewer monosaccharides in the LPS core were not only sensitive to CAMP and NHS but were avirulent on subcutaneous infecting mice and guinea pigs. This finding has confirmed that the LPS structure plays a key role in plague-caused lethality and that gene *gmhB*, alongside with the *waaC*, *hldE* and *waaA* genes found by us earlier [39], or the protein encoded by the *gmhB* gene may be considered a potential candidate for the role of molecular target for specific inhibitors of *Y. pestis* virulence.

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Conflict of Interest

The authors declare no conflict of interest.

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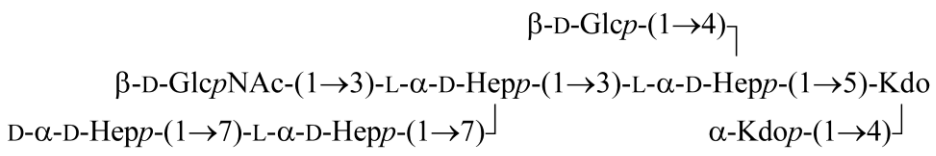
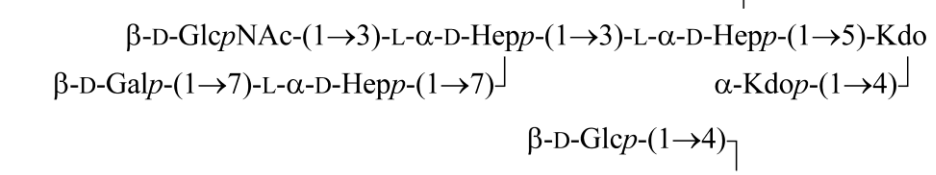
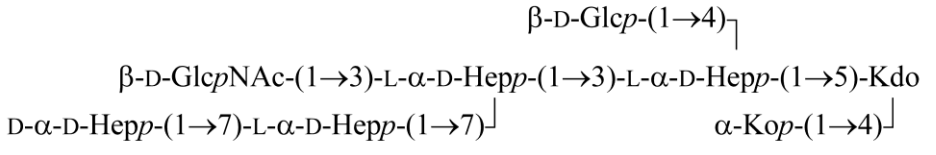
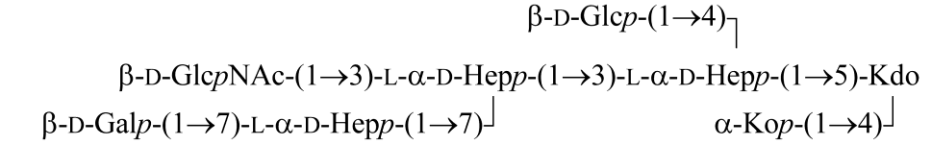
Table 1. Bacterial strains and plasmids used in this study

Strains and plasmids	Characteristics	Source or reference
Y. pestis KM218	pFra$^-$pCad$^-$pPst$^-$Δ*pgm*, derivative of the vaccine strain EV line NIIEG, avirulent	SCPB-Microbe
Y. pestis KM218-A3	*gmhB*::Tn*5*; obtained from strain KM218; Km^R	this study
Y. pestis KM218-A3/pAE-*gmhB*	KM218-A3, complemented with pAE-*gmhB*	this study
Y. pseudotuberculosis EV11M	serotype O:3; pCad$^-$	SCPM-Obolensk
E. coli S17-1 λ*pir*	*thi pro hsdR$^-$hsdM$^+$recA* RP4 2-Tc::Mu-Km::Tn*7*(Tp^{R}Sm^R)	[9]
E. coli S17-1 λ*pir*/pUT Km	transformant of the strain S17-1 λ*pir* with the plasmid pUT Km	this study
E. coli DH5α	F$^-$ϕ80*lacZ ΔM15 endA1 recA1 hsdR17 supE44 thi-1 gyrA96 relA1 (lacZYA-argF)U169*	[10]
pUT Km	*ori*R6K *bla tnp nptII*	[11] GenBank X06404
pUC19	*ori*pMB1 *bla* LacZ polylinker	[12] GenBank L09137
pACYC184	*ori*p15 *cat tet*	GenBank X06403
pAE-gfp	*ori*p15 *cat* SD-*gfp/asv*, derivative pACYC184	[13]
pAE-*gmhB*	Derivative pAE-gfp with *gmhB*	this study

Table 2. Sensitivity of *Yersinia* strains to PMB and NHS

Y. pestis strains	MIC of PMB (μg/ml)	Number of viable bacteria (l g CFU/ml) after incubation for 1 h in	
		NHS	HIS
Y. pestis KM218	500	6.31 ± 0.62	6.44 ± 0.61
Y. pestis KM218-A3	31.25	2.35 ± 0.22	6.42 ± 0.59
Y. pestis KM218-7	15.625	5.63 ± 0.49	6.85 ± 0.58
Y. pestis KM218-7K	31.25	5.82 ± 0.52	6.08 ± 0.64
Y. pestis KM218-45K	31.25	6.22 ± 0.59	6.35 ± 0.62
Y. pestis KM218-457K	62.5	0.54 ± 0.04	5.92 ± 0.00
Y. pestis KM218-804K	15.625	5.93 ± 0.57	6.55 ± 0.62
Y. pestis KM218-2048K	7.8	6.32 ± 0.61	6.44 ± 0.61
Y. pestis KM218-3209K	31.25	6.24 ± 0.60	6.47 ± 0.64
Y. pseudotuberculosis EV11M	7.8	0.74 ± 0.06	5.85 ± 0.54

Table 3. Types of core oligosaccharides in mutant strain *Y. pestis* KM218-A3

Core type	Structure of the core oligosaccharide
1a	
1b	
1c	
1d	
2a	
2c	
3a	
3c	

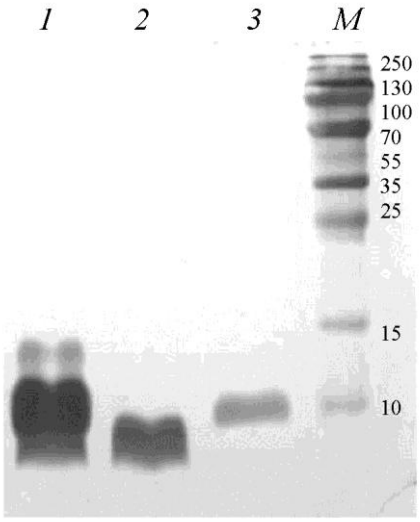


Fig. 1. SDS-PAGE of LPS preparations from the *Y. pestis* strains KM218-A3, KM218-A3/pAE-*gmhB*, and *Y. pseudotuberculosis* EV11M isolated by the method of Hitchcock and Brown [17]. Lanes: *1*) *Y. pestis* KM218-A3; *2*) *Y. pseudotuberculosis* EV11M; *3*) *Y. pestis* KM218-A3/pAE-*gmhB*; *M*) kit of standard proteins (Protein Molecular Weight Marker; Fermentas, Lithuania).

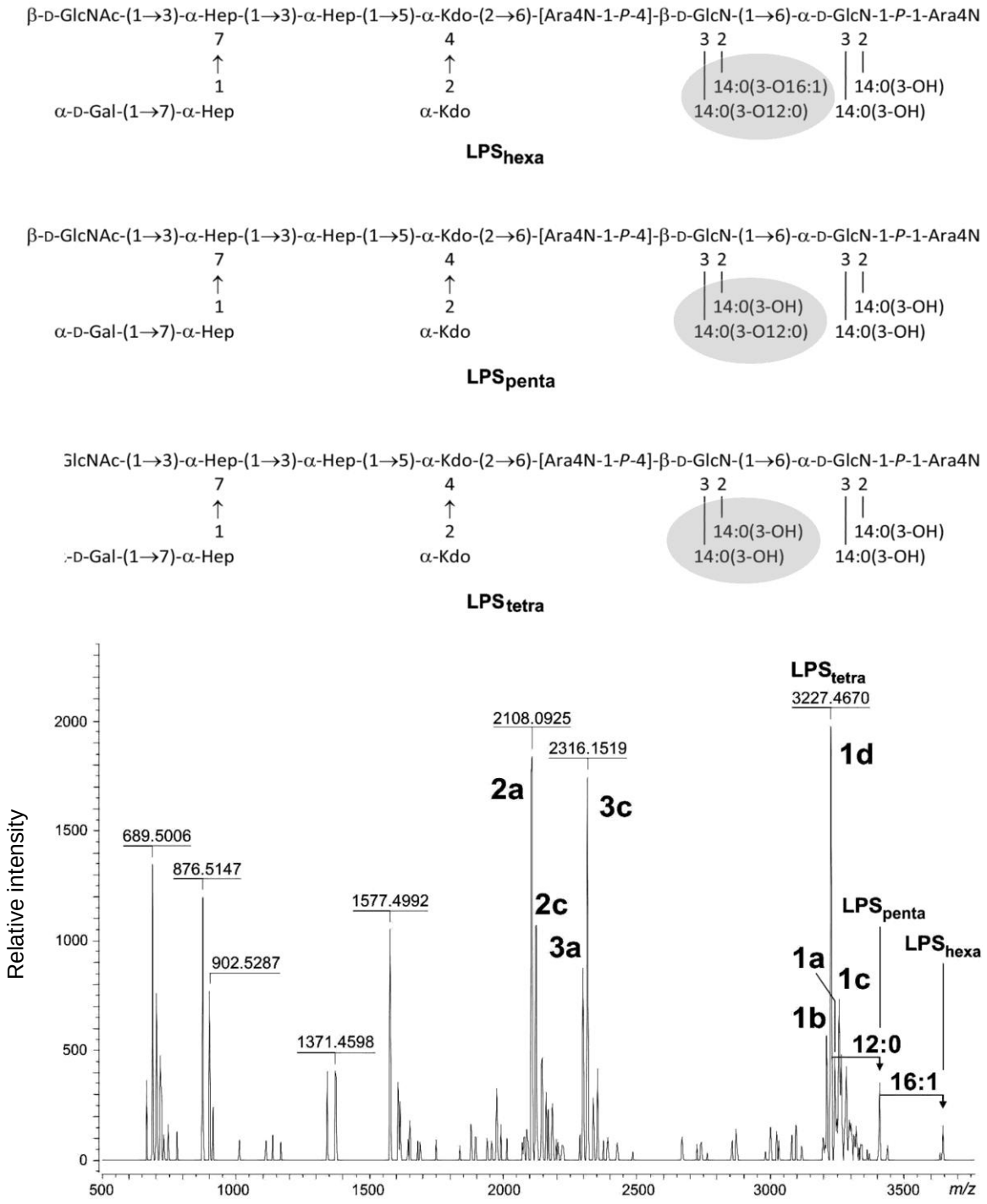


Fig. 2. ESI ICR FT mass-spectrum of anions derived from the unmodified LPS of *Y. pestis* strain KM218-A3 grown at temperature 25°C. Above the spectrum, the structures of hexacyl (LPS_{hexa}), pentacyl (LPS_{penta}), and tetracyl (LPS_{tetra}) forms of the LPS are shown. Ara4N, 4-amino-4-deoxy-L-arabinose; Hep, L-*glycero*-L-*manno*-heptose; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; 14:0(3-OH), (*R*)-3-hydroxymyristic acid; 14:0(3-O12:0), (*R*)-3-lauroyloxymyristic acid; 14:0(3-O16:1), (*R*)-3-palmitoleoyl-oxymyristic acid; *P*, phosphate group.

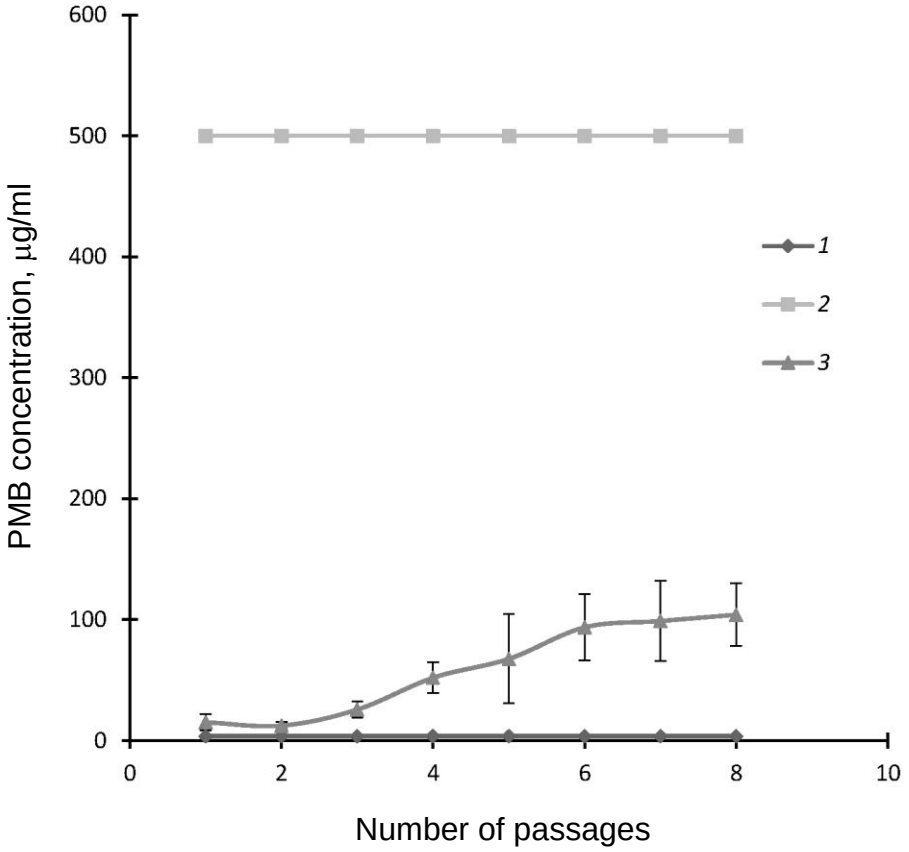


Fig. 3. Dependence of *Yersinia* PMB MIC on number of passages on solid nutrient medium: *1*) *Y. pseudotuberculosis* EV11M; *2*) *Y. pestis* KM218; *3*) *Y. pestis* KM218-A3.