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Effect of deletion of the *lpxM* gene on virulence and vaccine potential of *Yersinia pestis* in mice

Andrey P. Anisimov,[1] Rima Z. Shaikhutdinova,[1] Lyudmila N. Pan'kina,[2] Valentina A. Feodorova,[2] Elena P. Savostina,[2] Ol'ga V. Bystrova,[3] Buko Lindner,[4] Aleksandr N. Mokrievich,[1] Irina V. Bakhteeva,[1] Galina M. Titareva,[1] Svetlana V. Dentovskaya,[1] Nina A. Kocharova,[3] Sof'ya N. Senchenkova,[3] Otto Holst,[4] Zurab L. Devdariani,[2] Yuriy A. Popov,[2] Gerald B. Pier[5] and Yuriy A. Knirel[3]

Correspondence
Andrey P. Anisimov
anisimov@obolensk.org

[1]State Research Center for Applied Microbiology and Biotechnology, Obolensk 142279, Moscow Region, Russia

[2]Russian Research Anti-Plague Institute 'Microbe', Saratov 410071, Russia

[3]N. D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Moscow 119991, Russia

[4]Research Center Borstel, Leibniz Center for Medicine and Biosciences, D-23845 Borstel, Germany

[5]Channing Laboratory, Brigham and Women's Hospital, Harvard Medical School, Boston MA 02115, USA

Yersinia pestis undergoes an obligate flea–rodent–flea enzootic life cycle. The rapidly fatal properties of *Y. pestis* are responsible for the organism's sustained survival in natural plague foci. Lipopolysaccharide (LPS) plays several roles in *Y. pestis* pathogenesis, prominent among them being resistance to host immune effectors and induction of a septic-shock state during the terminal phases of infection. LPS is acylated with 4–6 fatty acids, the number varying with growth temperature and affecting the molecule's toxic properties. *Y. pestis* mutants were constructed with a deletion insertion in the *lpxM* gene in both virulent and attenuated strains, preventing the organisms from synthesizing the most toxic hexa-acylated lipid A molecule when grown at 25 °C. The virulence and/or protective potency of pathogenic and attenuated *Y. pestis* Δ*lpxM* mutants were then examined in a mouse model. The Δ*lpxM* mutation in a virulent strain led to no change in the LD_{50} value compared to that of the parental strain, while the Δ*lpxM* mutation in attenuated strains led to a modest 2.5–16-fold reduction in virulence. LPS preparations containing fully hexa-acylated lipid A were ten times more toxic in actinomycin D-treated mice then preparations lacking this lipid A isoform, although this was not significant ($P>0.05$). The Δ*lpxM* mutation in vaccine strain EV caused a significant increase in its protective potency. These studies suggest there is little impact from lipid A modifications on the virulence of *Y. pestis* strains but there are potential improvements in the protective properties in attenuated vaccine strains.

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INTRODUCTION

Yersinia pestis survives within an enzootic cycle involving infection of susceptible rodent populations and transmission via flea vectors (Anisimov, 1999a, 2002a, b; Brubaker, 1991; Hinnebusch, 2003, 2004; Lorange *et al.*, 2005; Perry, 2003; Perry & Fetherston, 1997). To maintain this cycle, *Y. pestis* must first cause a sustained and high-level bacteraemia in rodents that is sufficient to transfer the microbe to fleas following a blood meal (Anisimov, 1999a, 2002a; Brubaker, 1991; Feodorova & Golova, 2005; Hinnebusch, 2004; Lorange *et al.*, 2005; Perry & Fetherston, 1997). Next, *Y. pestis* must colonize the flea's alimentary canal producing a bacterial biofilm that blocks the proventriculus, the organ that connects the flea's oesophagus to its midgut. This blockage prevents the flea from taking a blood meal and forces it to bite a warm-blooded host repeatedly in a futile attempt to feed. At this stage, regurgitated bacteria enter the skin tissues at the site of the flea bite (Anisimov, 1999a, 2002a; Brubaker, 1991; Hinnebusch, 2003, 2004;

Abbreviations: CI, 95 % confidence interval; ESI FT-ICR, electrospray ionization Fourier transform ion cyclotron resonance; ImD_{50}, 50 % immunizing dose; LA_{hexa}, hexa-acyl lipid A; LA_{tetra}, tetra-acyl lipid A; LPS, lipopolysaccharide.

Lorange *et al.*, 2005; Perry, 2003; Perry & Fetherston, 1997). The bacteraemia caused by *Y. pestis* results from its impressive ability to overcome mammalian host defences and overwhelm hosts with massive growth (Anisimov, 2002a, b; Brubaker, 1991; Feodorova & Golova, 2005; Hinnebusch, 2004; Lorange *et al.*, 2005; Perry & Fetherston, 1997). Usually the bacteraemic phase evolves into lethal septic shock and the host's death forcing fleas to depart to feed on a new rodent, which subsequently becomes infected (Anisimov, 1999a, 2002a, b; Brubaker, 1991; Butler, 1989).

The triggering event of septic shock caused by Gram-negative bacteria is most likely the release of lipopolysaccharide (LPS). Lipid A, the toxic portion of the LPS molecule, causes the release of numerous host proinflammatory cytokines, and activates the complement cascade and the coagulation cascade. Recent studies suggest that Toll-like receptors, inflammatory cytokines, eicosanoids, free radicals, macrophage migration inhibitory factor, signal protein kinases and transcription factors all play an important part in the pathobiology of Gram-negative-mediated septic shock (Das, 2000; van Amersfoort *et al.*, 2003). *Y. pestis* LPS has no repeating O-antigen-like polysaccharide but is composed of lipid A and a temperature-dependent variable oligosaccharide analogous to the inner and outer core of enterobacterial LPS (Gremyakova *et al.*, 2003; Hitchen *et al.*, 2002; Kawahara *et al.*, 2002; Knirel *et al.*, 2005a, b; Prior *et al.*, 2001; Rebeil *et al.*, 2004; Vinogradov *et al.*, 2002). As it was shown for prototypic lipid A from *Escherichia coli*, its characteristic structural features, especially its two acyloxyacyl moieties and its two phosphate groups, are needed to trigger the endotoxin response in mammalian cells (Alexander & Rietschel, 2001; Rietschel *et al.*, 1994). Recently it was shown that in *Y. pestis* the increase from flea temperature (21–28 °C) to the host temperature (37 °C) caused a reduction of the immunostimulatory/endotoxic activity of LPS (Kawahara *et al.*, 2002; Rebeil *et al.*, 2004; Tynianova *et al.*, 2003) and a subsequent decrease of the degree of acylation of the lipid A from six to four fatty-lipid residues (Kawahara *et al.*, 2002; Knirel *et al.*, 2005a; Rebeil *et al.*, 2004).

lpxM (alternatively *msbB* or *waaN*) (Reeves *et al.*, 1996) is involved in the biosynthesis of lipid A. LpxM is a late-functioning acyltransferase for myristate (C_{14}), which functions optimally after laurate (C_{12}) incorporation by LpxL (alternatively HtrB or WaaM) (Reeves *et al.*, 1996) onto the *E. coli* KDO_2-lipid IV_A (2-keto-3-deoxyoctulosonic acid) structure (Clementz *et al.*, 1997; Raetz & Whitfield, 2002). Both LpxL and LpxM are the KDO-dependent acyltransferases responsible for the addition of a secondary acyl substitution on the lipid A portion of LPS (Brozek & Raetz, 1990; Clementz *et al.*, 1997; Nichols *et al.*, 1997; Raetz & Whitfield, 2002; Sunshine *et al.*, 1997). LPS isolated from Δ*lpxM* knockout mutants contains penta-acylated lipid A and *E. coli* Δ*lpxM* viable cells or purified LPS from this mutant had a 1000–10 000-fold reduction in the ability to stimulate E-selectin production by human endothelial cells and TNF-α production by adherent monocytes when compared with parent bacteria harbouring hexa-acylated lipid A (Somerville *et al.*, 1996). Recently it was shown that the *Y. pestis lpxM* (Dentovskaya *et al.*, 2006; Rebeil *et al.*, 2006) and *lpxP* (Rebeil *et al.*, 2006) homologues encode the acyltransferases that add C_{12} and $C_{16:1}$ groups, respectively, to lipid IV_A to generate the hexa-acylated form, and that their expression is upregulated at 21 °C *in vitro* and in the flea midgut (Rebeil *et al.*, 2006).

Recently it was shown that although LPSs from Δ*lpxM* mutants had decreased endotoxic activity, they were still as potent adjuvants of the immune response as hexa-acylated molecules from the parent strains (Steeghs *et al.*, 1999; van der Ley *et al.*, 2001) due to the equal ability of the hexa- and penta-acylated forms to upregulate surface molecule expression on dendritic cells and stimulate T-cells (Kalupahana *et al.*, 2003). Thus, a penta-acylated LPS isolated from a Δ*lpxL1* mutant of *Neisseria meningitidis* had decreased endotoxic activity but was still a potent adjuvant for immune responses (van der Ley *et al.*, 2001). These findings suggest that a *Y. pestis* Δ*lpxM* mutant also may be less virulent but may be useful for the development of new vaccines.

In this study, we investigated the effects of variations in the lipid A structure on the endotoxic activity of the LPS and on the pathogenicity of *Y. pestis* strains that differ in their initial virulence potential. To perform these investigations, we created Δ*lpxM* mutants in wild-type strain 231 as well as in attenuated strains, including the Russian vaccine strain, EV, line NIIEG. We also investigated the effect of a Δ*lpxM* mutation on the vaccine properties of strain EV. This mutation resulted in an inability to synthesize the hexa-acyl lipid A (LA_{hexa}) structure found in the parental strains when organisms are grown at 25 °C. Overall, we found a tenfold increase in the LD_{50} of the LPS from the Δ*lpxM* mutant, but this was not significant at $P > 0.05$. Inability of the virulent *Y. pestis* 231 Δ*lpxM* mutant to produce a hexa-acylated LPS did not change its ability to cause a lethal infection, while in the attenuated strains there was a modest reduction in virulence. Of note, in the vaccine strain, there was improved protective efficacy and decreased reactogenicity following immunization with the Δ*lpxM* mutant.

METHODS

Bacterial strains, plasmids and primers. The characteristics of the *Y. pestis* and *E. coli* strains used in this study are given in Table 1. *Y. pestis* strains were obtained from the Russian Anti-Plague Research Institute 'Microbe' (Russia) and were used in our previous studies as representatives of different biovars (Anisimov *et al.*, 2005; Knirel *et al.*, 2005a, b). Bacterial cultures were started from lyophilized stocks. For LPS isolation and structural analysis, to guarantee the safety of the investigators, *Y. pestis* strain 231 was cured of the pCD virulence plasmid by selection at 37 °C on magnesium oxalate agar plates (Higuchi & Smith, 1961). The variant was completely avirulent in mice at 10^8 organisms by the parenteral route. None of the absent plasmids (pFra, pCD, pPst) or missing parts of the genome (Δ*pgm*) of

Table 1. Bacterial strains used in this study

Strain	Description	Source or reference
Y. pestis		
231	Wild-type pFra$^+$ pCD$^+$ pPst$^+$; bv. antiqua, ssp. *pestis*; virulent*	Anisimov *et al.* (2004); Protsenko *et al.* (1983)
231Δ*lpxM*	pFra$^+$ pCD$^+$ pPst$^+$ Δ*lpxM*::*kan*; derived from the virulent strain 231; bv. antiqua, ssp. *pestis*; virulent	This study
KM260(11)	pFra$^+$ pCD$^-$ pPst$^-$; derived from the virulent strain 231; bv. antiqua, ssp. *pestis*; attenuated	Knirel *et al.* (2005a)
KM260(11)Δ*lpxM*	pFra$^+$ pCD$^-$ pPst$^-$ Δ*lpxM*::*kan*; derived from KM260(11); bv. antiqua, ssp. *pestis*; attenuated	This study
EV line NIIEG	pFra$^+$ pCD$^+$ pPst$^+$ Δ*pgm*; the Russian vaccine strain; bv. orientalis, ssp. *pestis*; attenuated	TSISCBP†
EVΔ*lpxM*	pFra$^+$ pCD$^+$ pPst$^+$ Δ*pgm* Δ*lpxM*::*kan*; derived from EV line NIIEG; bv. orientalis, ssp. *pestis*; attenuated	This study
KM218	pFra$^-$ pCD$^-$ pPst$^-$ Δ*pgm*; derived from EV line NIIEG; bv. orientalis, ssp. *pestis*; attenuated	Knirel *et al.* (2005a)
KM218Δ*lpxM*	pFra$^-$ pCD$^-$ pPst$^-$ Δ*pgm* Δ*lpxM*::*kan*; derived from KM218; bv. orientalis, ssp. *pestis*; attenuated	This study
KIMD1	pFra$^-$ pCD$^-$ pPst$^+$ Δ*pgm*; bv. medievalis, ssp. *pestis*; attenuated	Knirel *et al.* (2005a)
KIMD1Δ*lpxM*	pFra$^-$ pCD$^-$ pPst$^+$ Δ*pgm* Δ*lpxM*::*kan*; bv. medievalis, ssp. *pestis*; attenuated	This study
E. coli		
JM83	*ara* Δ(*lac-proAB*) *rpsL* ϕ80 *lacZ*ΔM15	Yanisch-Perron *et al.* (1985)
S17-1 λ*pir*	λ*pir* lysogen of S17-1 (*thi pro hsdR*$^-$ *hsd*M$^+$ *recA* RP4 2-Tc::Mu-Km::Tn*7*(Tp^R Sm^R)	Simon *et al.* (1983)

*For more detailed information on biovar-subspecies interrelations see Anisimov *et al.* (2004).
†Tarasevich State Institute for Standardization and Control of Biomedical Preparations, Sivtsev Vrazhek 41, Moscow 121002, Russia.

the mutant strains contained genes for LPS biogenesis, and did not exert influence on LPS structure when compared with the 'wild-type' in any of the strains (Hitchen *et al.*, 2002; Kawahara *et al.*, 2002; Knirel *et al.*, 2005a; Rebeil *et al.*, 2004) except for specially generated mutants (Δ*lpxM* mutants in this study). Plasmids and primers used are listed in Tables 2 and 3.

Medium and culture conditions. *E. coli* strains were routinely grown on Luria–Bertani (LB) agar or in LB broth (Miller, 1972) at 37 °C. For mutagenesis or virulence experiments, cultures of *Y. pestis* were grown at 25 °C in brain heart infusion (BHI) (Difco Laboratories) broth or on BHI agar. BHI agar with 5 % sucrose was used for the selection of *Y. pestis* double recombinants. For LPS isolation, *Y. pestis* strains were grown at 25 °C in New Brunswick Scientific fermenters with working volumes up to 10 l. Liquid aerated media was used and composed of fish-flour hydrolysate (20–30 g l^{-1}), yeast autolysate (10 g l^{-1}), glucose (3–9 g l^{-1}), K_2HPO_4 (6 g l^{-1}), KH_2PO_4 (3 g l^{-1}) and $MgSO_4$ (0.5 g l^{-1}), pH 6.9–7.1. pH and pO_2 control was used with the specified pO_2 value >10 %. The *Y. pestis* cellular biomasses were harvested by centrifugation, after 48 h of incubation, and then freeze-dried. Growth media were supplemented, as needed, with ampicillin (50 μg ml^{-1}), kanamycin (20 μg ml^{-1}) or polymyxin B (100 μg ml^{-1}).

Mutagenesis. A region 1529 bp 5′ (designated left shoulder) and a region 1500 bp 3′ (designated right shoulder) of the *lpxM* gene, including the first 180 nucleotides and the last 194 nucleotides, respectively (Table 3), were amplified by PCR with the use of a DNA template obtained from strain 231. The primers for the left shoulder, msb5S1.5 and msb3X, contained *Sac*I and *Xba*I restriction sites, respectively. The primers for the right shoulder, msb5H and msb3S1.5, contained *Hin*dIII and *Sac*I restriction sites, respectively (Table 3). To create the Δ*lpxM* mutants, the amplified left and right shoulders of the *Y. pestis lpxM* gene were gel purified, and cloned into the pBluescript SK (−) vector (Stratagene) within appropriate restriction sites. The resulting recombinant plasmids, pBsLS and pBsRS, respectively, were used to transform *E. coli* JM83 by electroporation. The cloned fragments of *Y. pestis lpxM* gene were then isolated following digestion with *Sac*I and *Hin*dIII and electrophoresis in a 0.9 % agarose gel. After that, they were assembled within the pUC19 vector digested with *Sac*I. The resulting recombinant plasmid, pUCLR, was used to transform *E. coli* JM83 by electroporation. This fragment, containing left and right shoulders of the *lpxM* gene with the polylinker *Xba*I–*Hin*dIII region from pBluescript SK (−) between them, was removed by digestion with *Sac*I and recloned within the *Sac*I site of the pCVD442 suicide vector. The resulting recombinant plasmid, pMSB8, was used to transform *E. coli* S17-1 λ*pir* by electroporation. A 1264 bp *Bam*HI fragment from pUC4K containing the Km^R gene was inserted in the *Bcu*I site at blunted ends in place of the deleted fragment of the *lpxM* gene (between the amplified shoulders of the *lpxM* gene). The resulting recombinant plasmid, pMSB3K, containing the indicated 5′ and 3′ flanking regions of the *lpxM* gene from *Y. pestis* 231 with a 0.59 kb deletion replaced by the Km^R locus from pUC4K, was electroporated into *E. coli* S17-1 λ*pir*.

The pMSB3K plasmid was introduced into *Y. pestis* strains by conjugation using polymyxin for counter-selection, the Km^R Ap^R exoconjugants were then counter-selected by growing on plates containing 5 % sucrose, and the Km^R Ap^S colonies in which allelic exchange had occurred were selected and deletion of the chromosomal *lpxM* gene confirmed by PCR (Donnenberg & Kaper, 1991).

Isolation of LPS and SDS-PAGE. LPSs were extracted from dried cells with phenol/chloroform/light petroleum ether (Galanos *et al.*, 1969) and purified by repeated ultracentrifugation (105 000 ***g***, 4 h), following enzymic digestion of nucleic acids and proteins. The purity of the isolated LPS preparations was evident from the lack of protein and nucleic acid contaminants as determined by SDS-glycine PAGE with silver staining of the gels (Prior et al., 2001). The LPS preparations from the *Y. pestis* KM218 parental and KM218Δ*lpxM* mutant strains were designated LPS_{parent} and $LPS_{\Delta lpxM}$, respectively.

Table 2. Plasmids used in this study

Plasmid	Description	Purpose	Antibiotic resistance	Source or reference
pBluescript SK (−)	High-copy-number cloning vector	Initial subcloning	Ap^R	Stratagene
pBsLS	A 1.5 kb *Sac*I-*Xba*I fragment containing left shoulder of *lpxM* in pBluescript SK (−)	Source of the left shoulder of *lpxM*	Ap^R	This study
pBsRS	A 1.5 kb *Hin*dIII-*Sac*I fragment containing right shoulder of *lpxM* in pBluescript SK (−)	Source of the right shoulder of *lpxM*	Ap^R	This study
pUC19	High-copy-number cloning vector	Subcloning of the both shoulders of *lpxM*	Ap^R	GenBank no. M77789
pUCLR	A 1.5 kb *Sac*I–*Xba*I fragment from pBsLS containing left shoulder of *lpxM* and *Xba*I–*Hin*dIII fragment of polylinker from pBluescript SK (−) + a 1.5 kb *Hin*dIII–*Sac*I fragment from pBsRS containing right shoulder of *lpxM* in pUC19 digested with *Sac*I	Source of the both shoulders of *lpxM*	Ap^R	This study
pCVD442	Suicide vector, *sacB*, *mob*	Suicide vector	Ap^R	Donnenberg & Kaper (1991)
pMSB8	A 3.0 kb *Sac*I fragment from pUCLR containing left and right shoulders of *lpxM* separated by *Xba*I–*Hin*dIII fragment of polylinker from pBluescript SK (−) in pCVD442	Subcloning into suicide vector	Ap^R	This study
pUC4K	Cloning vector, linker and kanamycin region	Source of Km^R cassette	Ap^R Km^R	GenBank no. X06404
pMSB3K	A 1.3 kb *Bam*HI fragment from pUC4K containing the Km^R gene in pMSB8	Subcloning into suicide vector for conjugation	Ap^R Km^R	This study

Mild acid degradation of LPS. Samples of LPS_{parent} and $LPS_{\Delta lpxM}$ were degraded with aqueous 2 % acetic acid at 100 °C for 4 h. The water-insoluble lipid precipitate (crude lipid A) from each sample was separated by centrifugation (12 000 ***g***, 15 min), washed and resuspended in water, lyophilized and the solid preparation then treated with a chloroform/methanol mixture (1 : 1, v/v), which extracts contaminating phospholipids from lipid A. The water-soluble supernatant was fractionated by gel-permeation chromatography on a column (70 × 2.6 cm) of Sephadex G-50 (S) (Amersham Biosciences) using aqueous 1 % aqueous acetic acid supplemented with 0.4 % pyridine as eluant. Monitoring was performed with a differential refractometer (Knauer). Fractions that contained core-like oligosaccharides were collected and lyophilized.

Mass spectrometry. High-resolution electrospray ionization Fourier transform ion cyclotron resonance (ESI FT-ICR) MS was performed in the negative ion mode using an Apex II instrument (Bruker Daltonics) equipped with a 7 T actively shielded magnet and an Apollo electrospray ion source as described previously (Knirel *et al.*, 2005a). Samples were dissolved at a concentration of ~10 ng μl^{-1} in a 50 : 50 : 0.001 (by vol.) mixture of 2-propanol/water/triethylamine, and sprayed at a flow rate of 2 μl min^{-1}. Capillary entrance voltage was set to 3.8 kV, and dry gas temperature to 150 °C. The spectra were charge deconvoluted, and mass numbers given refer to the monoisotopic molecular masses.

Animals. Outbred Swiss Webster mice weighing approximately 20 g were used in animal experiments that were approved by the ethical committee of the State Research Center for Applied Microbiology and Biotechnology. Animals were kept in cages in groups of four to eight, and allowed to feed and drink *ad libitum*. Median lethal doses (LD_{50}), 50 % immunizing doses (ImD_{50}) and 95 % confidence intervals (CI) were measured according to the method of Kärber (Ashmarin & Vorob'ov, 1962).

Testing the toxicity of LPS and the lethality of *Y. pestis* strains in mice. Actinomycin D-sensitized or naïve mice were used in our experiments to derive the LD_{50} values of LPS preparations and *Y.*

Table 3. Oligonucleotide primers used to amplify the 5′ and 3′ flanking regions of the *lpxM* gene

Gene amplified	Location (bp)*	Size (bp)	Product size (bp)	Primer	Sequence (5′ to 3′) and restriction site†
Left shoulder					
lpxM	2 340 356–2 340 384	29		msb5S1.5	ATGAGAGCTCCACCAAAGATGCCAATGAC, *Sac*I
lpxM	2 341 864–2 341 837	28	1529	msb3X	GGGCTCTAGAAGCAAATTTCCCAGCGAG, *Xba*I
Right shoulder					
lpxM	2 342 452–2 342 483	32		msb5H	TTATAAGCTTCATCGTCTTGATATCTATATCC, *Hin*dIII
lpxM	2 343 952–2 343 924	29	1500	msb3S1.5	AACAGAGCTCGCATCAACAAGTTAGGTGG, *Sac*I

*Relative to genome of *Y. pestis* CO92 (Parkhill *et al.*, 2001).

†Recognition sites for restriction endonucleases are underlined, and the restriction endonucleases indicated.

pestis attenuated strains under study. A total of 5 μg actinomycin D (AppliChem) in a total volume of 200 μl pyrogen-free 0.9 % NaCl solution was intraperitoneally injected into groups with 8 mice each along with tenfold dilutions of LPS preparations, or along with organisms obtained from cultures grown for 48 h at 25 °C (120 mice, divided into 30 groups of 4). Four groups of naïve mice (80 mice, divided into 20 groups of 4) were challenged subcutaneously (using tenfold dilutions) with suspensions of bacteria in 0.9 % NaCl solution in a total volume of 0.2 ml. Mortality due to LPS toxicity was recorded up to 7 days post injection, while animals infected with *Y. pestis* cultures were observed for a period of 21 days. Animals that succumbed to infection were dissected and bacterial cultures obtained to confirm systemic spread of *Y. pestis*.

Immunization and challenge. Bacterial cultures of either the EV line NIIEG strain or the EVΔ*lpxM* mutant were grown for 48 h at 25 °C and administered subcutaneously in a total volume of 0.2 ml 0.9 % NaCl solution (with tenfold dilutions from 10^9 to 10^5 c.f.u.) as a single injection on day 0 (70 mice, divided into 10 groups of 7). Two groups of seven mice were treated only with 0.9 % NaCl solution. At day 21 post-immunization the mice were challenged subcutaneously with 3.5×10^4 LD_{50} (2.1×10^5 c.f.u.) of *Y. pestis* strain 231. The two control groups of naïve animals were challenged subcutaneously with 3.5×10^4 LD_{50} (2.1×10^5 c.f.u.) or 17 LD_{50} (10^2 c.f.u.) of the same strain. The survival of mice was monitored daily for 21 days. The degree of protection offered by the vaccines was assessed by measuring the shift in the ImD_{50} values in the immunized animals.

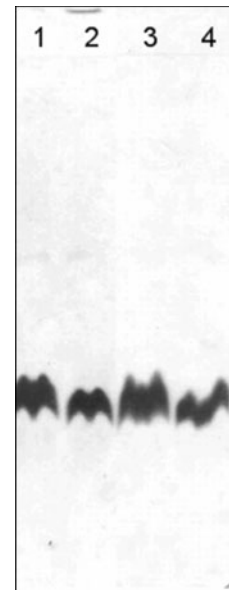

Fig. 1. Silver-stained SDS-PAGE gel of LPS samples from *Y. pestis* strains grown at 25 °C. *Y. pestis* samples (2.5 μg) were applied to the gel. Distinctions between the mobility of the LPS samples are seen most clearly when the upper confines of the bands are compared. Lane 1, KM218; lane 2, KM218Δ*lpxM*; lane 3, KIMD1; lane 4, KIMD1Δ*lpxM*.

RESULTS

Isolation and SDS-PAGE characterization of the LPSs

Each *Y. pestis* strain was grown at 25 °C and the corresponding LPS samples were isolated by phenol/chloroform/light petroleum extraction and fractionated by SDS-PAGE. Examples of LPS migration for two pairs of isogenic strains are shown in Fig. 1. LPS preparations from the Δ*lpxM* mutants migrated through the gel slightly faster and as more compact bands than those from the parent strains, suggesting that LPS molecules from the wild-type strains are, on average, larger.

Structural studies of the LPSs

LPS_{parent} and $LPS_{\Delta lpxM}$ from *Y. pestis* KM218 and KM260(11) were degraded under mild acid conditions to cleave the linkage between the core and lipid A. The isolated and purified lipid A and core oligosaccharide, as well as the whole LPS samples, were studied by ESI FT-ICR MS. The mass spectrum of the lipid A sample from *Y. pestis* KM218 LPS_{parent} (Fig. 2a) showed the predominance of tetra-acyl and hexa-acyl variants, whose structures have been determined previously (Aussel *et al.*, 2000; Knirel *et al.*, 2005a). LA_{hexa} includes four primary 3-hydroxymyristoyl groups (3OH14 : 0) and two secondary acyl groups, one dodecanoyl (12 : 0) and one palmitoleoyl (16 : 1); tetra-acyl lipid A (LA_{tetra}) is devoid of both secondary fatty acids, and minor penta-acyl lipid A variants ($LA1_{penta}$ and $LA2_{penta}$) lack either of them (Fig. 2e). Lipid A from *Y. pestis* KM218 $LPS_{\Delta lpxM}$ was distinguished by the absence of LA_{hexa} and $LA1_{penta}$, i.e. the structural variants that include the 12 : 0 group (Fig. 2b). Instead, a significant increase in the content of $LA2_{penta}$ with the single 16 : 1 secondary group was observed. Essentially the same structural variants were found when the mass spectra of the lipid A samples from *Y. pestis* KM260(11) LPS_{parent} and $LPS_{\Delta lpxM}$ were compared (Fig. 2c, d, respectively).

No significant changes in the core composition and the content of the cationic sugar, 4-amino-4-deoxy-L-arabinose (Ara4N), were observed in LPS_{parent} and $LPS_{\Delta lpxM}$ from both strains studied (data not shown). Therefore, our data confirmed the finding of Rebeil *et al.* (2006) that *Y. pestis* Δ*lpxM* mutants are unable to incorporate the dodecanoyl group into lipid A and showed that the other LPS biosynthesis pathways are unaffected by the mutation.

Toxicity of LPSs in actinomycin D-sensitized mice

Several substances (Galanos *et al.*, 1979; Seyberth *et al.*, 1972), including actinomycin D (Brown & Morrison, 1982; Seyberth *et al.*, 1972), when administered in sublethal amounts, have been documented to enhance by more than 1000-fold the sensitivity of mice to the lethal effect of LPS. Although the mechanisms of sensitization by these metabolic inhibitors have not been elucidated in detail, blocking of protein synthesis by inhibition of RNA synthesis may be involved. All animals challenged only with LPS-free actinomycin D solutions survived. All of the

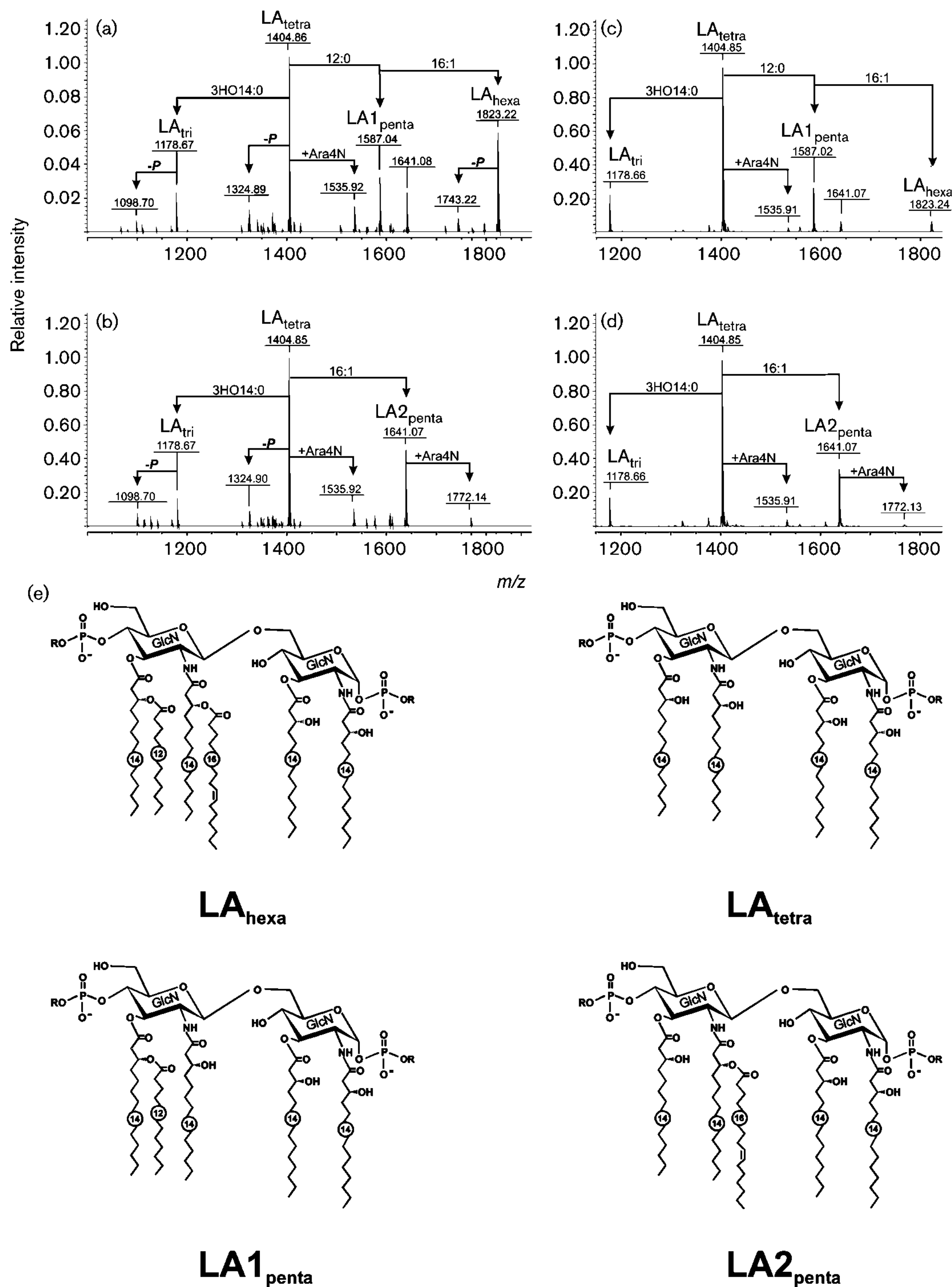

Fig. 2. Charge deconvoluted negative ion ESI FT-ICR mass spectra of lipid A samples from *Y. pestis* KM218 LPS$_{parent}$ (a), KM218 LPS$_{\Delta lpxM}$ (b), KM260(11) LPS$_{parent}$ (c), KM260(11) LPS$_{\Delta lpxM}$ (d), and structures of *Y. pestis* LA$_{hexa}$, LA$_{tetra}$ and penta-acyl lipid A variants (LA1$_{penta}$ and LA2$_{penta}$) (e). In the Δ*lpxM* mutants (b, d) no LA$_{hexa}$ nor LA1$_{penta}$ are detected. Ara4N, 4-Amino-4-deoxyarabinose. Numbers inside circles refer to numbers of carbons in fatty acids.

animals injected with LPS that died succumbed to endotoxic shock within 2 days of injection. The toxicity of the LPS preparation from the *Y. pestis* strain KM218 [LD_{50}=5 µg per animal (95 % CI=1–21 µg per animal)] was ten times as large as that from the Δ*lpxM* mutant [LD_{50}=53 µg per animal (95 % CI=13–337 µg per

animal)], but with the overlapping 95 % CI the differences in LD_{50} were not significant at $P>0.05$.

Affect of the deletion of *lpxM* on the lethality of virulent and attenuated *Y. pestis* strains in a murine system

The *Y. pestis* parental and Δ*lpxM* strains were used to assess the contribution to virulence made by having the genetic ability to synthesize a LA_{hexa}. There was no effect on virulence of the *Y. pestis* wild-type strain 231 due to deletion of *lpxM* (Table 4).

When attenuated strains (i.e. lacking the pCD plasmid or with further mutations) of *Y. pestis* are injected into mice, the residual virulence can be evaluated with either the use of very high infective doses or immunocompromised animals treated with immunosuppressants (Anisimov, 2002a; Brygoo & Rajenison, 1973; Jackson & Burrows, 1956; Vasil'eva *et al.*, 1988). In experiments with attenuated strains, the use of both approaches indicated that Δ*lpxM* mutation resulted in a 2.5–16-fold reduction in virulence as measured by an increase in LD_{50} (Table 4). The overlap in the 95 % CI for the LD_{50} for wild-type, and *lpxM*-deleted strains EV and KM218 indicated the increase in LD_{50} was not significant at $P>0.05$, but the one log increase in virulence for strain KIMD1 was significant at $P<0.05$. Thus loss of production of the hexa-acylated form of the LPS had a modest effect on virulence in these otherwise attenuated strains.

Influence of deletion of the *lpxM* gene on the protective potency of *Y. pestis* vaccine strains in a murine system

All of the mice immunized with doses of the Russian vaccine strain EV between 10^5 and 10^9 c.f.u. and challenged with 7×10^5 LD_{50} (4.2×10^6 c.f.u.) of wild-type, virulent *Y. pestis* strain 231 succumbed to infection, while 57 % of the animals immunized with comparable doses of vaccine strain with the *lpxM* gene deleted (EVΔ*lpxM*) survived. The calculated ImD_{50} values in immunized mice were $>10^9$ c.f.u. for the EV vaccine strain and 3.4×10^7 (95 % CI=8.6×10^6–1.3×10^8) c.f.u. for strain EVΔ*lpxM*. Analysis of the mean survival time indicated that immunization with EVΔ*lpxM* led to a 1.7-fold increase in survival time in animals immunized with this strain when compared with those immunized with the parental vaccine strain ($P<0.05$). Of note, when the challenge dose of the virulent *Y. pestis* strain 231 is reduced 20 times to 3.5×10^4 LD_{50} (2.1×10^5 c.f.u. of *Y. pestis* strain 231), as few as 2.4×10^3 c.f.u. (95 % CI=9.0×10^2–1.6×10^4) of the vaccine strain EV were able to prevent death of 50 % of the immunized animals (Anisimov, 1999b). Thus, the enhanced protective efficacy of the Δ*lpxM* mutant of the vaccine strain was only evident when a higher challenge inoculum of the virulent *Y. pestis* 231 was used.

DISCUSSION

Y. pestis easily overcomes the defence mechanisms of its animal hosts and readily proliferates in order to maintain the enzootic cycle essential to the organism's survival. To accomplish this, the organism must resist the innate immune response initiated by a variety of factors, including antimicrobial peptides and serum complement. The possibility to triumph over innate immunity response initiated by LPS-induced inflammatory cytokines is determined, in part, by the ability of the V antigen, which is a component of the type III secretion apparatus encoded on pCD, to provoke production of the anti-inflammatory cytokine, interleukin 10 (Brubaker, 2003). Production of other components of the type III secretion system that interfere with innate immune cellular factors and are cytotoxic for host cells are also essential for high-level virulence of *Y. pestis* (Cornelis, 2000). Resistance of Gram-negative bacteria to factors such as serum complement and antimicrobial peptides are generally related to carbohydrate components of the LPS, including both the monosaccharides present in the LPS oligosaccharide and 4-amino-L-arabinose in the lipid A (Anisimov *et al.*, 2005; Raetz &

Table 4. Virulence of the *Y. pestis* strains

Y. pestis strain	Application	LD_{50} c.f.u. (95% CI)	Mean time to death (days)*
231	Subcutaneous	6 (1–22)	4.9 ± 0.87 (3, 9)
231Δ*lpxM*		9 (2–38)	5.12 ± 0.62 (3, 8)
EV line NIIEG		6.3×10^7 (1.5×10^7–3.1×10^8)	4.29 ± 0.55 (3, 6)
EVΔ*lpxM*		2.9×10^8 (9.2×10^7–2.9×10^9)	4.0 ± 0.36 (3, 5)
EV line NIIEG	Intraperitoneal with actinomycin D (10 µg per mouse)	1.6×10^5 (4.0×10^4–6.3×10^5)	3.38 ± 0.77 (1, 6)
EVΔ*lpxM*		4.0×10^5 (1.0×10^5–1.6×10^6)	3.18 ± 0.64 (1, 5)
KM218		6.3×10^5 (1.5×10^5–2.5×10^6)	3.09 ± 0.69 (1, 5)
KM218Δ*lpxM*		4.0×10^6 (1.0×10^6–1.6×10^7)	2.37 ± 0.66 (1, 5)
KIMD1		1.0×10^5 (2.5×10^4–4.0×10^5)	3.5 ± 0.7 (1, 5)
KIMD1Δ*lpxM*		1.6×10^6 (4.0×10^5–6.3×10^6)	3.77 ± 1.05 (1, 7)

*The first and the last days of recorded deaths are given in parentheses.

Whitfield, 2002). The potency of LPS in regard to induction of inflammatory cytokines is determined by the overall composition and individual fatty acid constituents found in the lipid A (Kawahara *et al.*, 2002; Rebeil *et al.*, 2004; Raetz & Whitfield, 2002). A balanced production of variant forms of LPS in a pathogen such as *Y. pestis* that is dependent on the site and state of infection, and influenced by environmental conditions such as temperature (Kawahara *et al.*, 2002; Knirel *et al.*, 2005a; Rebeil *et al.*, 2004), seems to have significant evolutionary value in the context of maintenance of the organism's natural life cycle. We have, therefore, set out to determine how the described chemical variations in the lipid A of the *Y. pestis* LPS contribute to virulence.

Y. pestis produces an overall less-acylated lipid A at 37 °C that elicits less intense inflammatory responses, which seems to be beneficial for the pathogen by preventing rapid host responses leading to quick elimination following infection (Kawahara *et al.*, 2002). At temperatures below 26 °C, the more potent hexa-acylated LPS form predominates. Although it has been postulated that this LPS isoform may be necessary for survival during infection of fleas, it was recently shown by Rebeil *et al.* (2006) that there is no difference in the ability of a wild-type or a Δ*lpxM* Δ*lpxP Y. pestis* double mutant to survive in the flea digestive tract or to produce a transmissible infection. Therefore, at the present time a specific role for production of the hexa-acylated isoform of the LPS in the flea vector is not defined.

To determine the role of fatty-acid substitution on *Y. pestis* virulence in mammals, we generated a Δ*lpxM* mutant of the highly virulent *Y. pestis* strain 231, which was unable to incorporate the dodecanoyl group into lipid A. Cultures of the parent and mutant strains grown at 25 °C prior to infection showed no significant differences in their LD_{50} values. Thus, consistent with the findings of Rebeil *et al.* (2006) indicating there was no effect of LPS acylation on survival of *Y. pestis* in fleas, synthesis of the hexa-acylated LPS in arthropod vectors provided no advantage for *Y. pestis* during the initial stage of infection in the mammalian host. It remains to be determined if production of the hexa-acylated form of the LPS, which is increased at lower temperatures, is necessary for *Y. pestis* survival at the temperature of winter-hibernating rodents (6 °C).

In the case of attenuated *Y. pestis* strains, the Δ*lpxM* mutations resulted in a 2.5–16-fold reduction in residual virulence both in naïve and actinomycin D-sensitized animals. The same tenfold reduction in the toxicity of the $LPS_{\Delta lpxM}$ preparation in actinomycin D-sensitized mice points towards the idea that, at least in the animals sensitized to LPS, the reduction of virulence of Δ*lpxM* mutants was due to a decrease of $LPS_{\Delta lpxM}$ toxicity. This supposition was corroborated by the fact that the presence of a functional type III secretion system does not confer reliably increased virulence in actinomycin D-sensitized mice. In this assay the EV strain is only threefold more virulent than the KM218 strain, even though the latter is missing pCD.

While it does not appear that production of the hexa-acylated form of LPS is needed for virulence of wild-type *Y. pestis*, Δ*lpxM* mutants of other Gram-negative pathogens, such as *E. coli* (Somerville *et al.*, 1996), *Salmonella enterica* serovar Typhimurium (Khan *et al.*, 1998; Low *et al.*, 1999; Sunshine *et al.*, 1997), *Haemophilus influenzae* (Lee *et al.*, 1995; Nichols *et al.*, 1997), *Shigella flexneri* (D'Hauteville *et al.*, 2002), *Neisseria gonorrhoeae* (Post *et al.*, 2002), *N. meningitidis* (van der Ley *et al.*, 2001) and *Yersinia pseudotuberculosis* (Dentovskaya *et al.*, 2006), have reduced virulence. As these strains are less virulent than *Y. pestis*, it seems that in the absence of high levels of virulence, such as that which occurs in the plague bacillus, the *lpxM* gene does contribute to the organism's overall fitness to cause serious infections. Along the same lines, it was recently shown that even though the *S. enterica* serovar Typhimurium Δ*lpxM* mutant possesses less ability to induce production by dendritic cells of TNF-α, interleukin-1β and nitric oxide synthase than the parental strain (Kalupahana *et al.*, 2003), this effect was only seen at low multiplicities of infection ($\leqslant 0.5$). Infections at multiplicities of 5.0 or higher did not show any difference in the endotoxic properties of the two strains (Kalupahana *et al.*, 2003). Thus, the Δ*lpxM* mutants of highly virulent strains that can initiate clinical disease following inoculation with a low number of bacterial cells still can produce an endotoxic shock even when synthesizing a less toxic form of LPS, while Δ*lpxM* mutants of less virulent bacteria are more dependent on synthesizing a LPS with high endotoxic properties.

Two types of plague vaccines, live attenuated and killed whole-cell vaccines, are currently available for use in humans. Both vaccines cause generally mild reactions but sometimes reactions can be severe in a significant percentage of individuals immunized (Naumov *et al.*, 1992; Perry & Fetherston, 1997). The overwhelming majority of these reactions (Marshall *et al.*, 1974; Meyer *et al.*, 1974a, b; Naumov *et al.*, 1992; Reisman, 1970) are thought to be due to the bacterial endotoxin (Dmitrovskii, 1994; Galanos & Freudenberg, 1993; Morrison & Rayn, 1987; Raetz & Whitfield, 2002; van Amersfoort *et al.*, 2003; van der Poll & van Deventer, 1999). Given that the *Y. pestis* LPS does not elicit protective immunity (Feodorova *et al.*, 1999; Prior *et al.*, 2001) it is reasonable to minimize endotoxic components in plague vaccine preparations. To this end, a number of LPS-free subunit and naked DNA candidate vaccines have been reported to be relatively non-reactogenic. These subunit and DNA vaccines incorporate the F1 capsular and the LcrV (or simply the V) antigens or their genes, and they can provide significant protection against experimental plague infections (Titball & Williamson, 2001). However, since F1 capsule-negative *Y. pestis* strains have been recovered from at least one case of human infection and there is serological diversity in the V antigen, the current subunit vaccines under evaluation may

not be sufficiently comprehensive, particularly when considering that the *Y. pestis* strains found in North and South America are clonally derived from a single importation in the early 20th century, whereas in endemic plague foci in Russia and Asia there is considerably greater antigenic and genetic diversity (Anisimov *et al.*, 2004). While immunization with the live attenuated *Y. pestis* strain EV induces good protection against isolates with different antigenic compositions (Anisimov, 1999b) it is unlikely that one could generate viable LPS-free *Y. pestis* cells (Raetz & Whitfield, 2002; Rietschel *et al.*, 1994). However, by genetically manipulating the organism to only synthesize low-toxicity lipid A structures, it might be possible to produce a less reactogenic vaccine in an attenuated strain (van der Ley *et al.*, 2001).

In the countries of the former Soviet Union, live-vaccine-containing organisms grown at 28 °C that synthesize the hexa-acylated LPS are used for human immunization. This is due to the observation that *Y. pestis* cells grown at 28 °C are more viable and survive long-term storage better (Naumov *et al.*, 1992). However, growth at 28 °C decreases expression of the F1 antigen, although this did not apparently affect protection, as there were no differences observed in this parameter comparing individuals immunized with *Y. pestis* vaccine strain EV grown at 37 °C compared with that grown at 28 °C (Russell *et al.*, 1995; Sheremet *et al.*, 1987). Importantly, if deleting the *lpxM* gene renders the vaccine strain less reactogenic and more immunogenic, as was shown here in the mouse studies, this might be useful for producing an improved live *Y. pestis* vaccine strain for human use, as long as the differences in specificity of human and mouse LPS receptors (Delude *et al.*, 1995; Golenbock *et al.*, 1991) do not impact the decreased reactogenicity of the Δ*lpxM* mutant of *Y. pestis* in humans. Any of the attenuated *Y. pestis* strains showing high protective potency, including Pgm^- (Perry & Fetherston, 1997), Δ*dam* (Robinson *et al.*, 2005) and Δ*pcm* (Flashner *et al.*, 2004) mutants, may be used for further Δ*lpxM* knockout. Nevertheless, further work is required to construct a Δ*lpxM* vaccine candidate strain lacking antibiotic resistance for future preclinical trials.

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