Effect of deletion of the \textit{lpxM} gene on virulence and vaccine potential of \textit{Yersinia pestis} in mice

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\textit{Yersinia pestis} undergoes an obligate flea–rodent–flea enzootic life cycle. The rapidly fatal properties of \textit{Y. pestis} are responsible for the organism’s sustained survival in natural plague foci. Lipopolysaccharide (LPS) plays several roles in \textit{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. \textit{Y. pestis} mutants were constructed with a deletion insertion in the \textit{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 \textit{Y. pestis} \textit{DlpxM} mutants were then examined in a mouse model. The \textit{DlpxM} mutation in a virulent strain led to no change in the LD$_{50}$ value compared to that of the parental strain, while the \textit{DlpxM} 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 than preparations lacking this lipid A isoform, although this was not significant ($P>0.05$). The \textit{DlpxM} 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 \textit{Y. pestis} strains but there are potential improvements in the protective properties in attenuated vaccine strains.

INTRODUCTION

\textit{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 \textit{et al.}, 2005; Perry, 2003; Perry & Fetherston, 1997). To maintain this cycle, \textit{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 \textit{et al.}, 2005; Perry & Fetherston, 1997). Next, \textit{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;
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; Tyntianova 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 (C₁₄), which functions optimally after laurate (C₁₂) incorporation by LpxL (alternatively HtrB or WaaM) (Reeves et al., 1996) onto the E. coli KDO₂-lipid IVₐ (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 ΔlpxM knockout mutants contains penta-acylated lipid A and E. coli ΔlpxM 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 C₁₂ and C₁₆groups, respectively, to lipid IVₐ 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 ΔlpxM 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 ΔlpxL1 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 ΔlpxM 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 ΔlpxM 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 ΔlpxM mutation on the vaccine properties of strain EV. This mutation resulted in an inability to synthesize the hexa-acyl lipid A (LAhexa) structure found in the parental strains when organisms are grown at 25 °C. Overall, we found a tenfold increase in the LD₅₀ of the LPS from the ΔlpxM mutant, but this was not significant at P > 0.05. Inability of the virulent Y. pestis 231 ΔlpxM 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 ΔlpxM 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 10⁶ organisms by the parenteral route. None of the absent plasmids (pFra, pCD, pPst) or missing parts of the genome (Δpgm) of
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; Rebell et al., 2004) except for specially generated mutants (ΔlpxM 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), K2HPO4 (6 g l−1), KH2PO4 (3 g l−1) and MgSO4 (0.5 g l−1), pH 6.9–7.1. pH and pO2 control was used with the specified pO2 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, msbS15 and msb3X, contained SacI and XbaI restriction sites, respectively. The primers for the right shoulder, msbSH and msbS15, contained HindIII and SacI restriction sites, respectively (Table 3). To create the ΔlpxM 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, pBSL 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 SacI and HindIII and electrophoresis in a 0.9 % agarose gel. After that, they were assembled within the pUC19 vector digested with SacI. 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 XbaI–HindIII region from pBlueScript SK (+) between them, was removed by digestion with SacI and recloned within the SacI site of the pCVD442 suicide vector. The resulting recombinant plasmid, pMS88, was used to transform E. coli S17-1 Δpir by electroporation. A 1264 bp BamHI fragment from pUC4K containing the KmR gene was inserted in the BscI site at blunt ends in place of the deleted fragment of the lpxM gene (between the amplified shoulders of the lpxM gene). The resulting recombinant plasmid, pMS83K, 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 KmR locus from pUC4K, was electroporated into E. coli S17-1 Δpir.

The pMS83K plasmid was introduced into Y. pestis strains by conjugation using polymixin for counter-selection, the KmRe Ap+ exconjugants were then counter-selected by growing on plates containing 5 % sucrose, and the KmR Ap+ 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 KM218ΔlpxM mutant strains were designated LPSparent and LPSΔlpxM, respectively.

Table 1. Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y. pestis 231</td>
<td>Wild-type pFra+ pCD+ pPst+; bv. antiqua, ssp. pestis virulent*</td>
<td>Anisimov et al. (2004); Protosenko et al. (1983)</td>
</tr>
<tr>
<td>Y. pestis 231ΔlpxM</td>
<td>pFra+ pCD+ pPst+ ΔlpxM::kan; derived from the virulent strain 231; bv. antiqua, ssp. pestis</td>
<td>This study</td>
</tr>
<tr>
<td>KM260(11)</td>
<td>pFra+ pCD+ pPst−; derived from the virulent strain 231; bv. antiqua, ssp. pestis; attenuated</td>
<td>Knirel et al. (2005a)</td>
</tr>
<tr>
<td>KM260(11)ΔlpxM</td>
<td>pFra+ pCD+ pPst− ΔlpxM::kan; derived from KM260(11); bv. antiqua, ssp. pestis; attenuated</td>
<td>This study</td>
</tr>
<tr>
<td>EV line NIEEG</td>
<td>pFra+ pCD+ pPst+ Δpgm; the Russian vaccine strain; bv. orientalis, ssp. pestis attenuated</td>
<td>TSISCBP+</td>
</tr>
<tr>
<td>EVΔlpxM</td>
<td>pFra+ pCD+ pPst+ Δpgm ΔlpxM::kan; derived from EV line NIEEG; bv. orientalis, ssp. pestis attenuated</td>
<td>This study</td>
</tr>
<tr>
<td>KM218</td>
<td>pFra− pCD− pPst− Δpgm; derived from EV line NIEEG; bv. orientalis, ssp. pestis; attenuated</td>
<td>Knirel et al. (2005a)</td>
</tr>
<tr>
<td>KM218ΔlpxM</td>
<td>pFra− pCD− pPst− Δpgm ΔlpxM::kan; derived from KM218; bv. orientalis, ssp. pestis; attenuated</td>
<td>This study</td>
</tr>
<tr>
<td>KIMD1</td>
<td>pFra− pCD− pPst+ Δpgm; bv. medievialis, ssp. pestis; attenuated</td>
<td>Knirel et al. (2005a)</td>
</tr>
<tr>
<td>KIMD1ΔlpxM</td>
<td>pFra− pCD− pPst+ Δpgm ΔlpxM::kan; bv. medievialis, ssp. pestis; attenuated</td>
<td>This study</td>
</tr>
<tr>
<td>E. coli JM83</td>
<td>ara Δ(lac-proAB) rpsL 80 lacZAM15</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>S17-1 Δpir</td>
<td>Δpir lysogen of S17-1 (thi pro hsdR− hsdM+ recA RP4 2-Tc::Mu-Km::Tn7(TpR SmR)</td>
<td>Simon et al. (1983)</td>
</tr>
</tbody>
</table>

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

charides were collected and lyophilized.
refractometer (Knauer). Fractions that contained core-like oligo-
pyridine as eluant. Monitoring was performed with a differential
using aqueous 1 % aqueous acetic acid supplemented with 0.4 %
column (70
supernatant was fractionated by gel-permeation chromatography on a
extracts contaminating phospholipids from lipid A. The water-soluble
then treated with a chloroform/methanol mixture (1 : 1, v/v), which
were degraded with aqueous 2 % acetic acid at 100
u
Mild acid degradation of LPS.
Samples of LPSparent and LPSAlpxM
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 oligosac-
charides 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
transform ion cyclotron resonance (ESI FT-ICR) MS was performed
in the negative ion mode using an Apex II instrument (Bruker

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
Biotechnology. Animals were kept in cages in groups of four to eight,
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

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 LD50 values of LPS preparations and Y.

Table 2. Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Purpose</th>
<th>Antibiotic resistance</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBluescript SK</td>
<td>High-copy-number cloning vector</td>
<td>Initial subcloning</td>
<td>ApR</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pBSL</td>
<td>A 1.5 kb SacI-XbaI fragment containing left shoulder of lpxM in pBluescript SK (-)</td>
<td>Source of the left shoulder of lpxM</td>
<td>ApR</td>
<td>This study</td>
</tr>
<tr>
<td>pBSRS</td>
<td>A 1.5 kb HindIII-SacI fragment containing right shoulder of lpxM in pBluescript SK (-)</td>
<td>Source of the right shoulder of lpxM</td>
<td>ApR</td>
<td>This study</td>
</tr>
<tr>
<td>pUC19</td>
<td>High-copy-number cloning vector</td>
<td>Subcloning of the both shoulders of lpxM</td>
<td>ApR</td>
<td>GenBank no. M77789</td>
</tr>
<tr>
<td>pUCLR</td>
<td>A 1.5 kb SacI–XbaI fragment from pBSL containing left shoulder of lpxM and XbaI–HindIII fragment of polylinker from pBluescript SK (−) + a 1.5 kb HindIII–SacI fragment from pBSRS containing right shoulder of lpxM in pUC19 digested with SacI</td>
<td>Source of the both shoulders of lpxM</td>
<td>ApR</td>
<td>This study</td>
</tr>
<tr>
<td>pCVD442</td>
<td>Suicide vector, sacB, mob</td>
<td>Suicide vector</td>
<td>ApR</td>
<td>Donnenberg &amp; Kaper (1991)</td>
</tr>
<tr>
<td>pMSB8</td>
<td>A 3.0 kb SacI fragment from pUCLR containing left and right shoulders of lpxM separated by XbaI–HindIII fragment of polylinker from pBluescript SK (−) in pCVD442</td>
<td>Subcloning into suicide vector</td>
<td>ApR</td>
<td>This study</td>
</tr>
<tr>
<td>pUC4K</td>
<td>Cloning vector, linker and kanamycin region</td>
<td>Source of KmR cassette</td>
<td>ApR KmR</td>
<td>GenBank no. X06404</td>
</tr>
<tr>
<td>pMSB3K</td>
<td>A 1.5 kb BamHI fragment from pUC4K containing the KmR gene in pMSB8</td>
<td>Subcloning into suicide vector for conjugation</td>
<td>ApR KmR</td>
<td>This study</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Gene amplified</th>
<th>Location (bp)*</th>
<th>Size (bp)</th>
<th>Product size (bp)</th>
<th>Primer</th>
<th>Sequence (5’ to 3’) and restriction site†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left shoulder</td>
<td>lpxM</td>
<td>2 340 356–2 340 384</td>
<td>29</td>
<td>msb5S1.5</td>
<td>ATGAGAGCGTCCACCAAGATGCAATGAC, SacI</td>
</tr>
<tr>
<td>lpxM</td>
<td>2 341 864–2 341 837</td>
<td>28</td>
<td>1529</td>
<td>msb3X</td>
<td>GGGCTCTAGAAGCAATTTCCTCCAGCGAG, XbaI</td>
</tr>
<tr>
<td>Right shoulder</td>
<td>lpxM</td>
<td>2 342 452–2 342 483</td>
<td>32</td>
<td>msb5H</td>
<td>TTATAAGGTCTCTGCTTGATCTATATCC, HindIII</td>
</tr>
<tr>
<td>lpxM</td>
<td>2 343 952–2 343 924</td>
<td>29</td>
<td>1500</td>
<td>msb3S1.5</td>
<td>AACAGAGCCTGACATCAACACGTTAGTG, SacI</td>
</tr>
</tbody>
</table>

*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 naive 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 EVΔlpxM 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 10⁶ to 10⁵ 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 × 10⁶ LD₅₀ (2.1 × 10⁵ c.f.u.) of Y. pestis strain 231. The two control groups of naive animals were challenged subcutaneously with 3.5 × 10⁶ LD₅₀ (2.1 × 10⁵ c.f.u.) or 17 LD₅₀ (10² 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 ImD₅₀ values in the immunized animals.

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 ΔlpxM 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

LPSparent and LPSΔlpxM 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 LPSparent (Fig. 2a) showed the predominance of tetra-acyl and hexa-acyl variants, whose structures have been determined previously (Assuel et al., 2000; Knirel et al., 2005a). LAhexa 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 (LAtetra) is devoid of both secondary fatty acids, and minor penta-acyl lipid A variants (LA1penta and LA2penta) lack either of them (Fig. 2e). Lipid A from Y. pestis KM218 LPSΔlpxM was distinguished by the absence of LAhexa and LA1penta, i.e. the structural variants that include the 12 : 0 group (Fig. 2b). Instead, a significant increase in the content of LA2penta 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) LPSparent and LPSΔlpxM 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 LPSparent and LPSΔlpxM from both strains studied (data not shown). Therefore, our data confirmed the finding of Rebell et al. (2006) that Y. pestis ΔlpxM 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
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 [LD50 = 5 μg per animal (95% CI = 1–21 μg per animal)] was ten times as large as that from the ΔlpxM mutant [LD50 = 53 μg per animal (95% CI = 13–337 μg per...
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 ΔlpxM strains were used to assess the contribution to virulence made by having the genetic ability to synthesize a LA₆hexa. 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 ΔlpxM mutation resulted in a 2.5–16-fold reduction in virulence as measured by an increase in LD₅₀ (Table 4). The overlap in the 95 % CI for the LD₅₀ for wild-type, and lpxM-deleted strains EV and KM218 indicated the increase in LD₅₀ 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 the 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 10⁵ and 10⁹ c.f.u. and challenged with 7 x 10⁵ LD₅₀ (4.2 x 10⁶ 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 (EVΔlpxM) survived. The calculated ImD₅₀ values in immunized mice were >10⁹ c.f.u. for the EV vaccine strain and 3.4 x 10⁷ (95 % CI=8.6 x 10⁶–1.3 x 10⁸) c.f.u. for strain EVΔlpxM. Analysis of the mean survival time indicated that immunization with EVΔlpxM 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 x 10⁴ LD₅₀ (2.1 x 10⁵ c.f.u. of Y. pestis strain 231), as few as 2.4 x 10⁴ c.f.u. (95 % CI=9.0 x 10⁵–1.6 x 10⁶) 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 ΔlpxM 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>
<thead>
<tr>
<th>Y. pestis strain</th>
<th>Application</th>
<th>LD₅₀ c.f.u. (95% CI)</th>
<th>Mean time to death (days)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>231</td>
<td>Subcutaneous</td>
<td>6 (1–22)</td>
<td>4.9 ± 0.87 (3, 9)</td>
</tr>
<tr>
<td>231ΔlpxM</td>
<td></td>
<td>9 (2–38)</td>
<td>5.12 ± 0.62 (3, 8)</td>
</tr>
<tr>
<td>EV line NIIEG</td>
<td></td>
<td>6.3 x 10⁷ (1.5 x 10⁵–3.1 x 10⁶)</td>
<td>4.29 ± 0.55 (3, 6)</td>
</tr>
<tr>
<td>EVΔlpxM</td>
<td></td>
<td>2.9 x 10⁸ (9.2 x 10⁶–2.9 x 10⁸)</td>
<td>4.0 ± 0.36 (3, 5)</td>
</tr>
<tr>
<td>EV line NIIEG</td>
<td>Intraperitoneal with actinomycin D (10 µg per mouse)</td>
<td>1.6 x 10⁷ (4.0 x 10⁶–6.3 x 10⁷)</td>
<td>3.38 ± 0.77 (1, 6)</td>
</tr>
<tr>
<td>EVΔlpxM</td>
<td></td>
<td>4.0 x 10⁷ (1.0 x 10⁶–1.6 x 10⁷)</td>
<td>3.18 ± 0.64 (1, 5)</td>
</tr>
<tr>
<td>KM218</td>
<td></td>
<td>6.3 x 10⁵ (1.5 x 10⁵–2.5 x 10⁵)</td>
<td>3.09 ± 0.69 (1, 5)</td>
</tr>
<tr>
<td>KM218ΔlpxM</td>
<td></td>
<td>4.0 x 10⁶ (1.0 x 10⁵–4.0 x 10⁶)</td>
<td>2.37 ± 0.66 (1, 5)</td>
</tr>
<tr>
<td>KIMD1</td>
<td></td>
<td>1.0 x 10⁵ (2.5 x 10⁴–4.0 x 10⁵)</td>
<td>3.5 ± 0.7 (1, 5)</td>
</tr>
<tr>
<td>KIMD1ΔlpxM</td>
<td></td>
<td>1.6 x 10⁶ (4.0 x 10⁵–6.3 x 10⁶)</td>
<td>3.77 ± 1.05 (1, 7)</td>
</tr>
</tbody>
</table>

*The first and the last days of recorded deaths are given in parentheses.
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 ΔlpxM ΔlpxP 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 ΔlpxM 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 LD50 values. Thus, consistent with the findings of Rebeil et al. (2006) that 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 ΔlpxM 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 LPSΔlpxM preparation in actinomycin D-sensitized mice points towards the idea that, at least in the animals sensitized to LPS, the reduction of virulence of ΔlpxM mutants was due to a decrease of LPSΔlpxM 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, ΔlpxM 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 contributes 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 ΔlpxM 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 (≤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 ΔlpxM 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 ΔlpxM 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 \textit{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 \textit{et al.}, 2004). While immunization with the live attenuated \textit{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 \textit{Y. pestis} cells (Raetz & Whitfield, 2002; Rietschel \textit{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 \textit{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 \textit{Y. pestis} cells grown at 28 °C are more viable and survive long-term storage better (Naumov \textit{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 \textit{Y. pestis} vaccine strain EV grown at 37 °C compared with that grown at 28 °C (Russell \textit{et al.}, 1995; Sheremet \textit{et al.}, 1987). Importantly, if deleting the \textit{lpxM} gene renders the vaccine strain less reagentogenic and more immunogenic, as was shown here in the mouse studies, this might be useful for producing an improved live \textit{Y. pestis} vaccine strain for human use, as long as the differences in specificity of human and mouse LPS receptors (Delude \textit{et al.}, 1995; Golenbock \textit{et al.}, 1991) do not impact the decreased reagentogenicity of the \textit{ΔlpxM} mutant of \textit{Y. pestis} in humans. Any of the attenuated \textit{Y. pestis} strains showing high protective potency, including Pgm\textsuperscript{−} (Perry & Fetherston, 1997), \textit{Δdam} (Robinson \textit{et al.}, 2005) and \textit{Δpcm} (Flashner \textit{et al.}, 2004) mutants, may be used for further \textit{ΔlpxM} knockout. Nevertheless, further work is required to construct a \textit{ΔlpxM} vaccine candidate strain lacking antibiotic resistance for future preclinical trials.

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