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Pleiotropic effects of the *lpxM* mutation in *Yersinia pestis* resulting in modification of the biosynthesis of major immunoreactive antigens

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Abstract

Deletion mutants in the *lpxM* gene in two *Y. pestis* strains, the live Russian vaccine strain EV NIEG and a fully virulent strain, 231, synthesise a less toxic penta-acylated lipopolysaccharide (LPS). Analysis of these mutants revealed they possessed marked reductions in expression and immunoreactivity of numerous major proteins and carbohydrate antigens, including F1, Pla, Ymt, V antigen, LPS, and ECA. Moreover, both mutants demonstrated altered epitope specificities of the antigens as determined in immunodot-ELISAs and immunoblotting analyses using a panel of monoclonal antibodies. The strains also differed in their susceptibility to the diagnostic plague bacteriophage L-413C. These findings indicate that the effects of the *lpxM* mutation on reduced virulence and enhanced immunity of the *Y. pestis* EV $\Delta lpxM$ is also associated with these pleiotropic changes and not just to changes in the lipid A acylation.

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Keywords

Yersinia pestis EV NIEG; *Yersinia pestis* 231; *lpxM(msbB)* mutant; live plague vaccine; plague immunity; virulence; *Yersinia pestis* antigens

1. Introduction

The live plague vaccine used in Russia and elsewhere is based on the *Y. pestis* strain EV line NIEG, which is a derivative of an attenuated pigmentation-negative strain, *Y. pestis* EV76. This strain has been widely used for protection of plague researchers, other humans living in territories endemic for plague in Russia and other countries of the Former Soviet Union (FSU) [1-5] and also in camel [6]. The properties of the *Y. pestis* EV line NIEG and other *Y. pestis* EV76 derivatives have been carefully studied using different animal models, and a number of distinctive immunobiological characteristics of this live plague vaccine have been reported [1-4,7-18]. The main feature of this vaccine is its ability to induce a relatively rapid (on day 7 after immunisation) and high level of specific immunity against both bubonic and pneumonic plague following by a single injection. The reversion of the *Y. pestis* EV76 vaccine to a fully virulent form has not been described [3,7-9] as attenuation is due to the spontaneous deletion of c.a. 102-kb “pigmentation” (Pgm) region encoding the hemin-storage (*hms*) locus and high pathogenicity island (HPI) [19]. The live EV NIEG plague vaccine showed superior protective properties over other live and killed plague vaccines developed thus far [3,5,7,8,20]. The main factor limiting the worldwide licensing of this live vaccine EV NIEG is the fact that this vaccine possesses a ‘residual virulence’ [3,4,11,15,20] and can occasionally trigger undesirable local and systemic reactions in immunised individuals, likely mediated by the *Y. pestis* endotoxin [5,9,20-25].

Recently, we reported that a mutation made in the *Y. pestis* EV line NIEG genome leading to inactivation of the acyltransferase gene *lpxM* resulted in synthesis of a less toxic penta-acylated lipopolysaccharide (LPS), whereas a more toxic hexa-acylated lipid A is produced when bacteria are grown at 25 °C [20,22,23,25,26]. Using three animal models we showed that this *lpxM* mutant displayed improved characteristics as a vaccine, such as decreased endotoxic activity and overall reactogenicity, and enhanced protective immunity in comparison with the parental vaccine strain [20]. Likely, the decreased adverse effects of the mutant can be directly attributed to the production of less toxic LPS. However, it is not apparent why the mutant possessed improved protective properties. One of the possible explanations for increased protective immunity of the *lpxM* mutant could be an altered expression of major immunoreactive antigens that might result in modification of their presentation to the host immune system. Indeed, deleting the *lpxM* gene in other bacterial pathogens often leads to pleiotropic effects, resulting in membrane alterations and attenuation in virulence [27-32]. For example, in addition to marked reductions in LPS toxicity, the *lpxM* mutant (also known as *waaN* or *msbB*) [33,34] of the clinical *Escherichia coli* H16 isolate had reduced synthesis of the K1 capsular material leading to an increase in complement C3 deposition on the cell surface, enhancement in both opsonic and nonopsonic phagocytosis, and the appearance of a filamentous phenotype [27]. Deletions of the *lpxM* gene significantly reduced virulence of other bacterial pathogens as well, including *Salmonella enterica* serovar Typhimurium [28,32,35]. These bacteria were elongated, formed bulges, grew slowly and a certain part of cell population could form filaments [28]. In addition, this mutant had an impaired ability to stimulate synthesis of tumor necrosis factor α (TNF- α) and interleukin-1 β as well as inducible nitric oxide synthase both *in vitro* and *in vivo* [32,35]. Similar structural modifications in the lipid A of *Neisseria meningitidis* also resulted in reduction of LPS toxicity and adjuvant activity, affected the lipooligosaccharide assembly and transport of outer membrane porins [29]. The *lpxM*

mutant of the *E. coli* O157:H7 possessed increased susceptibility to antibiotics and detergents as well as altered bacterial motility, formation of curli and Shiga toxin production [31]. Similarly, the *Klebsiella pneumoniae* strain B5055 Δ *lpxM* producing primarily a penta-acylated lipid A demonstrated an increased permeability of the outer membrane (OM), an elevated susceptibility to certain antibacterial peptides (polymyxin B, colistin, polymyxin E, CP28 and C18G) and had only approximately half of total synthesis of uronic acid capsule important in providing resistance to human complement [36]. However, there is no absolute correlation between deletions of the *lpxM* gene and a pleiotropic phenotype or reduction in virulence in all pathogenic bacteria investigated thus far. For instance, in *Shigella flexneri*, a Δ *lpxM* derivative had no defects either in growth, division or morphology [30], nor did deletion of this gene have noticeable influence on the virulence of the wild-type strain *Y. pestis* 231 suggesting a limited contribution of this lipid A modification in virulence of the plague bacterium [25]. Moreover, it was not clear whether the *lpxM* mutation in *Y. pestis* led to alternations of other membrane components or properties in both vaccine and virulent *Y. pestis* strains or to the consequent modification in the production of major immunoreactive antigens nor how these changes might correlate with the molecular mechanisms leading to protection against plague.

In this study, we carefully investigated the *Y. pestis lpxM* mutants for the synthesis and immunoreactivity of major known antigens involved in virulence of *Y. pestis* and development of immunity against plague. The results of this analysis showed the marked reduction in expression and immunoreactivity as well as changes in the epitope specificity of major surface proteins and carbohydrate antigens in the *Y. pestis lpxM* mutant that was accompanied by alterations in the corresponding phenotypic activities.

2. Materials and methods

2.1. Bacterial strains and cultural conditions

The vaccine strain *Y. pestis* EV NIEG (Pgm⁻), fully virulent *Y. pestis* 231 (Pgm⁺) and their *lpxM* derivatives have been described previously [20,25]. The *Y. pestis* KM218 strain, is a plasmid-less isogenic derivative of the vaccine strain *Y. pestis* EV NIEG [37,38] which was used as a control to study the expression and immunoreactivity of the *Y. pestis* antigens (F1, Pla, Ymt, etc.) by immunoblotting. The strains were cultured stationary in Hottinger broth, pH 7.2, either at 28 °C for 48 h or at 28 °C for 24 h and then at 37 °C for 24 h.

2.2. Determination of bacterial capsule-like substance and F1 antigen production

To identify the ability of the cells of *Y. pestis* EV NIEG and its Δ *lpxM* derivative to form a capsule-like substance, the bacteria were analysed by electron microscopy as described elsewhere [39]. For this purpose, bacterial samples were examined with a JEOL model JSM-U3 microscope. Test samples of bacterial suspensions were placed on carbon-coated collodion grids followed by the treatment and negative staining as described [39].

The expression and immunoreactivity of the capsular antigen F1 (Caf1) of the *Y. pestis* EV NIEG, the *Y. pestis* 231 and their Δ *lpxM* derivatives were tested in a dot-ELISA with monoclonal antibodies (MAb) produced by the hybridoma cell line Y.p.F1.B₂.D₃.Sp. (MAb-F1) as reported previously [39]. Also, the production of the F1 antigen was determined by an indirect hemagglutination test with polyclonal antibodies specific to the *Y. pestis* capsular fraction F1 (PAb-F1) (The Institute for Microbiology of the Ministry of Defense, Kirov, Russia) using a routine technique [40]. At least six individually grown cultures of each strain were analysed in this test. The F1 antigen used to obtain PAb-F1 represented a crude preparation of *Y. pestis* capsule isolated by the method of Serdobintsev *et al.* [38,41].

2.3. Plasminogen activator activity testing

The fibrinolytic and plasma-coagulase activities of plague plasminogen activator protease (Pla) for the *Y. pestis* EV NIEG, the *Y. pestis* 231 and their $\Delta lpxM$ derivatives were assayed according to a routine technique [40].

To study fibrinolytic activity, a suspension of 1×10^9 cells/ml of each of the *Y. pestis* strains in 0.25 ml of a 0.9% saline solution was mixed with native citrated rabbit plasma (0.5 ml), diluted 1:10 in a 0.9% saline solution (v/v) and a 0.5% solution of calcium chloride (0.1 ml). The mixture was incubated for 20 h at 37 °C with subsequent visual evaluation of the degree of dissolution of a clot at 1, 2, 4 and 20 h after beginning of the assay. The plasma mixed with both 0.9% saline solution and calcium chloride solution at the same concentrations was used as a control.

To study the *Y. pestis* plasma-coagulase activity, the bacterial cells of either *Y. pestis* parental strains or their $\Delta lpxM$ mutants (each at a concentration 0.5×10^9 cells/ml) were incubated in native citrated rabbit plasma (0.5 ml), diluted 1:10 in a 0.9% saline solution, for 24 h at 28 °C. Uninfected plasma, diluted 1:10 in a 0.9% saline solution, was used as a control. The formation of the clot was determined by visual inspection after 1, 2, 4 and 20 h.

The production of the Pla by *Y. pestis* cells was estimated in dot-ELISA with a MAb to the Pla (MAb-Pla) as described previously [39,42].

2.4. Carbohydrate epitope immunoreactivity testing

To study the immunoreactivity of the carbohydrate epitopes of the *Y. pestis* EV NIEG and its $\Delta lpxM$ mutant, we used a panel of MAbs directed to *Y. pestis* or *Y. pseudotuberculosis* species-specific or shared epitopes. The specificity of the MAbs is shown in Table 1.

The MAb 1A₆ (MAb-1A₆) which recognises the *Y. pestis* core-lipid A epitope widely present in *Y. pestis* strains independent of their geographical isolation and temperature of cultivation has been previously characterised in detail [43]. Other MAbs of the panel were described recently [44]. Among the MAbs found to be species-specific for *Y. pestis*, MAbs 1D₁₀, 2D₇ and 6D₁₁ which recognised the epitopes on the enterobacterial common antigen (ECA) of *Y. pestis* [45] and MAbs 1G₈, 1F₈, 2D₁₁, 2E₄, 1C₄ 1B₇ and 3C₇ bound to galactolipid epitopes [38,44]. In regard to the effect of growth temperature on MAb binding, MAb 1G₈ was able to recognise *Y. pestis* bacteria grown either at 28 °C or at 37 °C, whereas MAbs 1F₈ and 6D₁₁ only bound to bacteria grown at 37 °C. MAb 4B₁₁ was species-specific for *Y. pseudotuberculosis* grown at either 28 °C or 37 °C, and MAbs 1B₇ and 5E₆ were immunoreactive with both *Y. pestis* and *Y. pseudotuberculosis* grown at 28 °C only. The MAbs 1C₄ and 3F₇ recognised the epitopes of *Y. pseudotuberculosis* grown at 28 °C and did not react with the cells of the *Y. pestis* EV NIEG.

2.5. Detection of the *Y. pestis* V antigen production

The production of V antigen by *Y. pestis* strains EV NIEG and 231 as well as their $\Delta lpxM$ derivatives was assayed in indirect dot-ELISA with the relevant MAb (MAb-V) which has been characterised previously [46].

2.6. SDS-PAGE and immunoblotting

These methods were used to identify a production of the capsular antigen F1 as well as Pla, Ymt and V antigens by the *Y. pestis* EV NIEG strain and its $\Delta lpxM$ derivative. For this purpose, whole-cell lysates of the *Y. pestis* strains (5×10^8 cells/lane) were resolved in 12.5% SDS-PAGE along with protein mol. wt. markers. The gels were either counterstained with Coomassie Brilliant Blue R-250 (Sigma) or electro-transferred to nitrocellulose

membranes (NCM) (Schleier-Schuell) for immunoblot analysis [38]. In the latter case, the membranes were incubated with a commercial plague polyclonal antibodies (PPA) raised in horses (Russian State Anti-plague Research Institute 'Microbe', Saratov, Russia), which contain antibodies specific for the *Y. pestis* F1, Pla, Ymt and several other *Y. pestis* antigens [39,47]. Specific bands were visualised with peroxidase-labeled rabbit anti-horse IgG (N.F. Gamaleya Scientific Research Institute for Epidemiology and Microbiology, Russian Academy of Medical Sciences, Moscow, Russia) using 3,3'-Diaminobenzidine tetrachloride (DAB) as a substrate. To identify the protein bands corresponding to the *Y. pestis* F1, Pla, Ymt and V antigens, the membranes were incubated with the relevant MABs to each of the antigens (MAB-F1, or MAB-Pla, or MAB-Ymt or MAB-V) as was reported previously [38,39,42,47].

SDS-PAGE was also used to study the carbohydrate profiles of the *Y. pestis* EV NIEG and its *lpxM* derivative. For this purpose, proteinase K-treated (PK-treated) whole-cell lysates of these strains were subjected to SDS-PAGE as described above. After that, the gels were counterstained using the silver-staining technique [48] or Coomassie Brilliant Blue R-250 as a control to visualise the proteinase K-resistant proteins.

2.7. Immunoreactivity with PPA

To study the combined immunoreactivity of the F1, Pla and Ymt antigens, the *Y. pestis* EV NIEG and 231 strains as well as their *lpxM* mutants were assayed in dot-ELISA using the PPA as described previously [39].

2.8. Evaluation of antibody responses following immunisation with live *Y. pestis* EV NIEG and its *lpxM* derivative

Different groups of 6-8-week-old female outbred mice (n = 10) were inoculated subcutaneously with 0.2 ml of a 0.9% saline solution containing 1.0×10^7 colony forming units (CFU) of either the *Y. pestis* EV NIEG or *Y. pestis* EV $\Delta lpxM$ strains grown at 28 °C for 48 h. A single group of negative control mice (n = 3) received 0.2 ml of a 0.9% saline solution alone. Mice were bled on day 28 post-immunisation. The antiserum of each of mouse was tested in indirect ELISA. For this purpose, ELISA plates (96-well) were sensitised with 1 µg/well of either capsular antigen F1 isolated by the method of Serdobintsev *et al.* [41] or LPS of *Y. pestis* grown at 28 °C and isolated by the method of Galanos *et al.* [49]. In addition, a sonicated cell extract (1.0×10^7 CFU/well) of the *Y. pestis* EV NIEG strain grown either at 28 °C or 37 °C was obtained by using an Ultrasonic Homogeniser CP501 (Cole Parmer, USA) and the lysate then used to sensitise the ELISA plates. After immobilisation of antigen, the plates were blocked with 1% bovine serum albumin (BSA) in PBS for 1 h at 37 °C and washed three times with washing buffer (PBS containing 0.05% of Tween-20). Then, two-fold serial dilutions of sera starting from a 1:20 dilution were applied in triplicate, the plates were incubated for 1 h at 37 °C then washed three times as described above. Peroxidase-labeled rabbit anti-mouse IgG (Sigma, St. Louis, MO) diluted 1:10,000 in a blocking buffer of 1% BSA in PBS were added, and the plates were incubated for 1 h at 37 °C followed by six washes. O-phenylenediamine (Sigma) was used as a substrate. The plates were incubated for 30 min at room temperature followed by the measurement of absorbance at optical density (OD) of 492 nm. The antibody titer was calculated as the highest dilution of the triplicate means that gave an OD of 3 standard deviations above the negative control.

2.9. Stimulation of TNF-α production by the macrophage-like cell line J774.A1 in response to treatment with different types of *Y. pestis* LPS

The macrophage-like J774.A1 and fibrosarcoma L929 murine cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of fetal bovine

serum in 5% CO₂ environment. To stimulate production of TNF- α , different concentrations of LPS were added to the monolayer of the J774.A1 cells (10⁶ cells) followed by the incubation for 24 h and consequent recovery of supernatants. Preparation of hexa-, penta- and tetra-acylated LPS from *Y. pestis* strain KM218, a plasmid-less derivative of EV NIEG, was described by us previously [25]. We used *E. coli* O55:B5 (Sigma) LPS and LPS of vaccine strain of *F. tularensis* 15/10 isolated by the method of Westphal *et al.* [50] as positive and negative controls, respectively. To determine the amount of TNF- α produced by the LPS-stimulated J774.A1 cells, the supernatants collected at the 24 hour time point were used to treat 10⁵ cell/ml of the indicator murine fibrosarcoma cell line L929 which is sensitive to TNF- α . The TNF-mediated cytotoxicity of L929 cells was evaluated by addition at 24 hours post-treatment of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at concentration of 5 μ g/ml followed by incubation for 4 hours [51]. The precipitated dye was dissolved with 0.04 N HCl in isopropanol and the absorbance in the triplicate wells was measured at an optical density of 540 nm using an ELISA plate reader (LabSystem Multiscan, Finland). Serial dilutions of recombinant murine TNF- α (Sigma) were used for obtaining a calibration curve to determine the quantities of TNF- α in the samples which were estimated in the scope of pg/ml.

2.10. Bacteriophage susceptibility test

The bacterial cells of the *Y. pestis* 231 Δ *lpxM* strain as well as cells of the parental *Y. pestis* 231 strain, used as a positive control (*ca.* 7×10^8 cells), were assayed with the diagnostic plague bacteriophages L-413C and Pokrovskaya [38,52] using a routine technique as described previously for the *Y. pestis* EV Δ *lpxM* [20]. All colonies obtained during construction of the *lpxM* mutant by allelic exchange were resistant to the phage L-413C, but sensitive to the Pokrovskaya phage. The parent *Y. pestis* 231 strain was sensitive to both phages.

3. Results

3.1. Changes in the immunoreactivity of the capsular antigen F1 in the *Y. pestis* Δ *lpxM* mutants

The *Y. pestis* capsular antigen F1 has previously been found to be a protective immunogen, but it is not required for full virulence of *Y. pestis* in mice, guinea pigs and African green monkeys [53-55]. However, the capsule of the plague microbe is involved in the development of resistance to phagocytosis, facilitating establishment of the *Y. pestis* infection [39,56,57]. We also previously showed that the *Y. pestis* EV Δ *lpxM* strain demonstrated enhanced immunity against bubonic plague in comparison with the *Y. pestis* EV NIEG vaccine [20], but deletion of the *lpxM* gene in the wild-type *Y. pestis* 231 strain had no effect on virulence [25]. To address a possible correlation between the level of synthesis of the capsular antigen F1 and enhanced immunogenic properties of the *Y. pestis* Δ *lpxM* mutants, we carefully tested the production and immunoreactivity of the F1 antigen expressed by both of the Δ *lpxM* derivatives of the *Y. pestis* EV NIEG and 231 strains. In addition, the formation of a capsule-like envelope was studied by electron microscopic analysis in the *Y. pestis* EV NIEG and its *lpxM* mutant.

Both *Y. pestis* parental strains EV NIEG and 231 reacted in indirect haemagglutination test with the monospecific PAb-F1 with similar titers (1:1,024), whereas both of the Δ *lpxM* derivatives had reduced titers (Fig. 1). The *Y. pestis* EV NIEG Δ *lpxM* strain showed only a two-fold decrease in the titers of the reaction with the *Y. pestis* capsular antigen F1, but the *Y. pestis* 231 Δ *lpxM* strain demonstrated almost 64-fold reduction. Data indicating additional changes had occurred in the F1 expression by both Δ *lpxM* mutants was obtained in a dot-ELISA with extracts probed with either PPA or the MAb-F1 antibodies. The cells of both *Y.*

pestis parental strains demonstrated a similar intensity of the color signal with both polyclonal and monoclonal antibodies to F1, and the reaction was relatively weaker in the cells grown at 28 °C than with bacteria cultured at 37 °C (Table 2). In contrast to the reactivity to the polyclonal antibody in the indirect hemagglutination assay, there was no visual reaction of the *Y. pestis* EV NIEG $\Delta lpxM$ strain with the MAb-F1 directed to the Caf1 protein subunit, suggesting that the mutant had a change in the capsular antigen resulting in loss of reactivity with the MAb. In general, the decrease in F1 production by the *Y. pestis* EV NIEG $\Delta lpxM$ strain could be confirmed by using PPA as well, since the majority of antibodies in this serum are specific to the capsular antigen. Indeed, when PPA was used, the $\Delta lpxM$ mutant produced a positive, but weaker signal than that of the parent *Y. pestis* EV NIEG. Similar observations were made for the *Y. pestis* 231 strain and its $\Delta lpxM$ derivative, and in contrast to the parental strains, the *Y. pestis* 231 $\Delta lpxM$ cells were found to be more immunoreactive with both MAb-F1 and PPA when the bacteria were grown at 28 °C than when grown at 37 °C.

We next determined the level of production of the major Caf1 subunit in the wild-type and $\Delta lpxM$ mutant of *Y. pestis* EV NIEG to determine if alterations in antigen expression were present in the mutant. For this purpose, we compared the whole-cell lysates of the *Y. pestis* EV NIEG and its $\Delta lpxM$ mutant by SDS-PAGE and immunoblotting analyses. As expected, the SDS-PAGE revealed that the Caf1 with mol.wt 18.5±2 kDa was clearly seen in the whole-cell lysates of the *Y. pestis* EV NIEG strain, but as a minor protein band when the bacteria were grown at 28 °C and as a major band after growth of the bacteria at 37 °C (Fig. 2a, lanes 1, 3, respectively). In contrast, the Caf1 protein could not be visualised in the whole-cell lysates of the *Y. pestis* EV $\Delta lpxM$ grown at either 28 °C or 37 °C (Fig. 2a, lanes 2, 4). The other envelope components, i.e. 14-kDa, 28-kDa, 43-kDa, 60-kDa and 70-kDa proteins described previously [38,58] were readily found in *Y. pestis* EV NIEG and its $\Delta lpxM$ derivative. Both the 43-kDa and the 60-kDa peptides were seen as prominent protein bands of similar intensity when comparing wild-type and $\Delta lpxM$ *Y. pestis* strains. A marked difference was revealed in two other peptides. Both a 28-kDa and a 70-kDa proteins were visualised in the *Y. pestis* EV NIEG parent strain as minor bands when cells were grown at 28 °C (Fig. 2a, lane 1) and as major bands when bacteria were grown at 37 °C (Fig. 2a, lane 3). In the *Y. pestis* EV NIEG $\Delta lpxM$ mutant both of these peptides were detected as minor bands independent of the temperature of cultivation of the bacteria (Fig. 2a, lanes 2, 4). The 14-kDa protein was identified only in the *Y. pestis* EV NIEG grown at 37 °C (Fig. 2a, lane 3). Each of these proteins was identified in immunoblotting with the relevant MAb (data not shown) as described elsewhere [38].

To determine more thoroughly the changes in production of the capsular substances/cell envelope components, lysates from the *Y. pestis* EV NIEG strain and its $\Delta lpxM$ derivative, along with purified Caf1 protein, were probed in an immunoblot with commercial PPA which contains antibodies to a number of *Y. pestis* proteins. The strongest signal was observed for the protein band corresponding to the monomeric 18.5±2 kDa Caf1 subunit. The other reactive proteins were those with mol/wt of 28±2 kDa (minor band), 43±2 kDa (major band), 60±2 kDa (major band) and 70±2 kDa (major band). The 28-kDa and 43-kDa proteins which were shown to be encoded chromosomally [38,59] could also be visualised in the whole-cell lysate of the plasmid-less *Y. pestis* KM218 strain, an isogenic derivative of the *Y. pestis* EV NIEG [37,38]. Immunoblot analysis of the *Y. pestis* EV $\Delta lpxM$ whole-cell lysate revealed that the both 43-kDa and 60-kDa peptides had similar signal intensities for the protein bands in comparison to those of the parent strain. In contrast, no specific reaction with the Caf1 antibodies, as well as a clear decrease in the expression of both the 28-kDa and 70-kDa peptides were observed in the *Y. pestis* EV $\Delta lpxM$ whole-cell lysate (data not shown), indicating a significant reduction in expression of Caf1 and other surface antigens of the mutant.

Transmission electron microscopy with negative staining revealed that the *Y. pestis* EV NIEG parental bacteria grown at 37 °C had a strongly pronounced and distinct gel-like capsular envelope around the cell body (Fig. 3, b). No capsular substance was seen when the bacteria were grown at 28 °C (Fig. 3, a). The *Y. pestis* EV $\Delta lpxM$ cells possessed only a marginal irregular shape on the periphery of the cell body which was formed independently of the growth temperature. There was no capsular substance detected even when the higher resolution microscopy was used (Fig. 3, e, d).

3.2. Changes in the Pla activity of the *Y. pestis* $\Delta lpxM$ mutants

Pla is a cell surface-located protease of *Y. pestis*, with versatile virulence-associated functions that contains a three-dimensional motif for protein binding to lipid A [59]. This virulence factor is crucial for dissemination of the pathogen within the host, evasion of the host inflammatory immune response *in vivo* in both bubonic [59-62] and pneumonic plague [63] as well as for *in-vitro* host-pathogen interaction in conditions mimicking mammalian extracellular environment [39].

We therefore examined the immunoreactivity of Pla expressed by *Y. pestis* cells and found that there were marked changes in this feature in the *lpxM* mutants in comparison with the parental strains. Both *Y. pestis* parental strains interacted similarly with the MAb-Pla in dot-ELISA and demonstrated a typical strong positive reaction with bacteria grown at 37 °C and markedly weaker interaction with the same bacteria cultured at 28 °C (Table 2). The analysis of the interaction of MAb-Pla with the *Y. pestis* EV $\Delta lpxM$ revealed a weak positive reaction of this mutant independent of the temperature of cultivation of the bacteria. In contrast, the *Y. pestis* 231 $\Delta lpxM$ mutant possessed a definite enhanced immunoreactivity in comparison with the parental *Y. pestis* 231, indicating differential effects of the *lpxM* loss in these two different genetic backgrounds.

Since virulence-associated properties of Pla depend on its fibrinolytic and coagulase activities [40,60,61,63] we compared these characteristics in the parent and *lpxM* mutant strains. *Y. pestis* cells expressing Pla activated plasminogen and dissolved the clot. Tubes containing cells of the *Y. pestis* EV NIEG bacteria (as well as in control tubes with no bacterial cells), the clot was formed within 1 hour, and then completely dissolved after 20 hours of incubation at 37 °C (Table 3). In contrast, a delay in clot formation was noticed when the *Y. pestis* EV $\Delta lpxM$ cells were used, and this phenomenon was more pronounced for the *Y. pestis* strains 231 and *Y. pestis* 231 $\Delta lpxM$. Moreover, in these samples, the clot was lysed only partially after 20 hours of incubation (Table 3). In general, there was no significant difference between the parental strain and corresponding *lpxM* mutant in plasma coagulation assay (Table 3). Each strain of *Y. pestis* was able to induce a coagulation of plasma after 4 hours of incubation at 28 °C, although strain 231 and its derivative were more efficient in this reaction. No clot formation was seen in the control tubes with the plasma alone without *Y. pestis* cells.

The Pla protease can appear on SDS-PAGE gels as a band with mol. wt. ~ 35 kDa corresponding to a precursor form, as well as a band with mol. wt. ~ 33 kDa of the mature protein (α -Pla) and its slightly smaller auto-processed form with mol. wt. ~30 kDa (β -Pla) [59,64,65]. We found both 33 and 30 kDa peptides as major bands in the *Y. pestis* EV NIEG grown at 28 °C, but only the 30-kDa peptide as a major band and the 33-kDa one as a minor band were detected in the *Y. pestis* EV NIEG grown at 37 °C (Fig. 2a, lanes 1, 3, respectively). This observation is in good correlation with previously reported data that β -Pla accumulates as a dominant form in the outer membrane of *Y. pestis* [66]. In contrast, both the α -Pla and the β -Pla forms were seen as major bands in the *Y. pestis* EV $\Delta lpxM$ mutant at both temperatures of cultivation indicating the decline in the processing of Pla is likely behind the reduced proteolytic activity of Pla made by this strain. These results were

confirmed by immunoblot with PPA, which revealed the immunoreactivity of both the α -Pla and β -Pla forms of Pla (data not shown).

3.3. Reduction of the production of V antigen by the *Y. pestis* Δ lpxM mutants

We determined whether the mutation in the *lpxM* gene had any influence on synthesis of the V antigen, a multifunctional virulence factor and protective antigen of *Y. pestis* [66,67]. The *lpxM* mutants of the both *Y. pestis* strains EV NIEG and 231 demonstrated a visible decrease in immunoreactivity with the MAb-V in dot-ELISA in comparison with that of the parent strains (Table 2). The observation that the production of the V antigen was reduced by the mutant strains was confirmed by SDS-PAGE of the whole-cell lysates of the *Y. pestis* EV NIEG and its *lpxM* derivative grown at 37 °C. As shown in Figure 2a (lane 3), the 38-kDa V antigen was visualised as a strong protein band in the samples from the parent strains. In contrast, this antigen was seen as a minor band in the whole-cell lysate of the *Y. pestis* EV Δ *lpxM* grown at the same temperature (Fig. 2a, lane 4). As expected, this antigen was barely produced by both strains of *Y. pestis* at 28 °C (Fig. 2a, lanes 1 and 2).

3.4. Changes of the Ymt expression in the *Y. pestis* Δ lpxM mutants

In our previous study we showed that the *Y. pestis* EV Δ *lpxM* was less toxic when used for immunisation of animals (mice, guinea pigs) as a live vaccine [20]. Likely, the reduced toxicity can be attributed to the synthesis by the mutant of the less toxic penta-acylated LPS, however, it might be possible that the production of other toxic components of *Y. pestis* has also been changed. One of the substances which could be considered in this category is a plague murine toxin, Ymt, which is highly toxic for mice and rats [5,40]. Therefore, we tested whether Ymt was expressed differently in the *lpxM* mutant and its parent strain, and found that the biosynthesis of the Ymt was reduced in the *Y. pestis* EV Δ *lpxM*. Indeed, SDS-PAGE revealed the Ymt as a major protein band with mol. wt 61 $\pm$ 2 kDa in the whole-cell lysate of the *Y. pestis* EV NIEG grown at 37 °C (Fig. 2a, lane 3) although the mutant strain produced this protein at a significantly reduced level (Fig. 2a, lane 4). Similarly, immunoblotting analysis with PPA confirmed that the production of Ymt was decreased in the mutant strain (data not shown).

3.5. Modification of the expression of additional proteins in the *Y. pestis* EV Δ lpxM mutant

To study possible changes in the production of other proteins by the *lpxM* mutants, the whole-cell lysates of the *Y. pestis* EV Δ *lpxM* bacteria grown either at 28 °C or at 37 °C were compared with those of the parental *Y. pestis* EV NIEG strain in SDS-PAGE. We found in the *Y. pestis* EV NIEG grown at 37 °C (Fig. 2a, lane 3), but not at 28 °C (Fig. 2a, lane 1), at least four thermoinducible protein bands with mol. wt 24.0 $\pm$ 2, 30.9 $\pm$ 2, 33.9 $\pm$ 2 and 55.0 $\pm$ 2 kDa, which were not detected in the *Y. pestis* EV Δ *lpxM* grown at either temperature (Fig. 2a, lanes 3, 4). At the same time, in the Δ *lpxM* strain grown either at 28 °C or at 37 °C, a 68.0 $\pm$ 2-kDa protein was detected which was also found in the *Y. pestis* EV NIEG strain grown at 28 °C that did not appear in the sample of the parental *Y. pestis* EV NIEG strain cultured at 37 °C. Additionally, three proteins with mol. wt 87.1 $\pm$ 2, 89.1 $\pm$ 2 and 93.3 $\pm$ 2 kDa were seen in the *Y. pestis* EV NIEG, grown at 37 °C as major bands while these proteins were represented as minor bands in its *lpxM* mutant (Fig. 2a, lanes 2 and 4). Our overall evaluation of the protein content of the *Y. pestis* Δ *lpxM* suggests that the mutant produced lower spectrum of proteins than its parental strain.

3.6. Modification of immunoreactivity of the surface located carbohydrate epitopes in the *Y. pestis* EV NIEG Δ lpxM mutant

A temperature-dependent change in the *Y. pestis* lipid A structure from a potent immunostimulatory hexa-acylated form produced at ambient temperature to a weak immuno-

stimulatory tetra-acylated molecule at mammalian host temperature is considered to be an essential factor which enables *Y. pestis* to evade the adaptive host immune response [21,24,25,39]. The analysis of LPS preparations isolated from the *Y. pestis* $\Delta lpxM$ revealed that the cells of the mutant synthesised a less toxic penta-acylated LPS as judged by SDS-PAGE and mass-spectroscopy [25]. The acylation pattern of the lipid A can potentially lead to modifications in the immunoreactivity of the epitopes among the carbohydrate antigens including the *Y. pestis* LPS. To address this assumption, we tested the immunoreactivity of both *Y. pestis* EV $\Delta lpxM$ mutant and the *Y. pestis* EV NIEG parental strain by using a panel of fourteen MABs with different epitope specificities. Also, the carbohydrate profiles of the PK-treated whole-cell lysates of these two strains were compared in the SDS-PAGE.

We found in dot-ELISA a marked difference in immunoreactivity of the carbohydrate epitopes of *lpxM* mutant and the *Y. pestis* EV NIEG parental strain. The four MABs (1A₆, 4B₁₁, 5E₆ and 1B₇) out of 14 used interacted identically (either positive or negative reaction) with the cells of both *Y. pestis* EV $\Delta lpxM$ and the *Y. pestis* EV NIEG bacteria (Table 1). Three of these four MABs were directed to epitopes on the lipid A-core part of LPS [43,44] of either *Y. pestis* (MAB 1A₆) or *Y. pseudotuberculosis* (MABs 4B₁₁ and 5E₆) indicating the absence of significant modification of this moiety of the LPS molecule between the mutant and parent strains. Other eight MABs demonstrated altered immunoreactivity with the mutant cells. Four MABs (3F₇, 2E₄, 1C₄ and 3C₇) showed a vigorous positive reaction with cells of the $\Delta lpxM$ derivative while there was no interaction with the parental strain (Table 1). This fact indicated that the epitopes recognised by these MABs may normally be masked in the *Y. pestis* EV NIEG parent strain but become exposed and available for MAB binding in the mutant cells. In contrast, the two MABs (1G₈, 1F₈) which reacted with the *Y. pestis* EV NIEG failed to interact with the *Y. pestis* EV $\Delta lpxM$; another MAB (2D₁₁) which was also directed to the galactolipid of *Y. pestis* was able to recognise the mutant strain, but only when the cells were grown at 28 °C (Table 1). Similarly, the MABs 1D₁₀, 2D₇ and 6D₁₁ specific to the ECA of *Y. pestis* reacted with the parent, but not with the mutant strain. The importance of this observation could be viewed from the fact that the bacterial ECA is directly involved in bacterial recognition by the host immune system [68].

By SDS-PAGE analysis of the PK-treated whole-cell lysates of either the *Y. pestis* EV $\Delta lpxM$ strain or the *Y. pestis* EV NIEG parental strain grown at 28 °C or 37 °C there were no overall gross differences found in the carbohydrate profiles nor in the intensity of staining (Fig. 2b). An alteration was observed in the intensity of a single intermediate-molecular-mass component which became stronger by changing from light-brown to dark-brown color in the *lpxM* derivative independent of the cultivation temperature. Also, the size of predominant lower-molecular-mass component was slightly increased and a single additional band migrating in front of it was detected in the lysate of *Y. pestis* EV $\Delta lpxM$ grown at 37 °C (Fig. 2b, lane 4). No protein bands were detected in the same gels after their staining with Coomassie Brilliant Blue indicating that there were no the proteinase K-resistant proteins in the PK-treated whole-cell lysates.

3.7. Effect of *lpxM* mutation on antibody response to the *Y. pestis* EV NIEG vaccine

We previously showed that immunisation of animals (mice, guinea pigs) with live cells of the *Y. pestis* EV $\Delta lpxM$ significantly increased the level of protection against *s.c.* challenge with fully virulent *Y. pestis* 231 in comparison with the parental vaccine strain *Y. pestis* EV NIEG [20]. To determine whether the increase in protective potential of the *lpxM* mutant could be at least partially assigned to the difference in humoral response between two strains, we determined murine antibody titers to capsular antigen F1, LPS and sonicated cell extracts of *Y. pestis* EV NIEG grown at 28 °C and 37 °C.

As shown in Fig. 4, the titer to F1 was significantly higher in animals which received inoculation with EV Δ *lpxM* cells in spite of their altered production of this antigen (Table 2 and Fig. 1). A similar observation was made when the F1-rich cell extract of EV NIEG cells cultivated at 37 °C was used to sensitise ELISA plates. The difference in titers elicited to both strains was negligible when the cell extract of EV NIEG grown at 28 °C (Fig. 4) or *Y. pestis* LPS (data now shown) were used as an antigenic target. The phenomenon of increased antibody response to the F1 antigen whose production was actually reduced in the EV Δ *lpxM* strain likely could be attributed to the marked adjuvant, but not toxic activity, characteristic to the penta-acylated LPS. For instance, a significant adjuvant activity in mice has been demonstrated for the LPS of *lpxM* mutants of *N. meningitidis* [29].

3.8. Penta-acylated LPS possessed a reduced capacity of stimulating TNF- α in vitro

The ability of LPS isolated from most microorganisms to stimulate production of TNF- α followed by treatment of macrophage-like cell line J774.A1 is a well-established phenomenon. In general, the level of induction of this cytokine correlates with the endotoxic activity of LPS attributed to its lipid A portion. Therefore, we compared the LPS of *Y. pestis* with different type of acylation of the lipid A, such as penta-acylated purified from the cells of *lpxM* mutant cultivated at 28 °C as well as hexa-acylated and tetra-acylated LPS isolated from the parental strain grown at 28 °C and 37 °C, respectively. We found, that the stimulating potency of hexa-acylated LPS was high and similar to that of control LPS of *E. coli* O55:B5 (Fig. 5). As expected, the tetra-acylated LPS of *Y. pestis* had a decreased ability to induce the production of TNF- α . Nevertheless, the stimulating potency of this type of LPS was superior over that of LPS of *F. tularensis*, known for its weak endotoxic activity. Similarly to the tetra-acylated LPS, the stimulating activity of the penta-acylated LPS was also reduced (Fig. 5).

3.9. Sensitivity of *Y. pestis* 231 Δ *lpxM* mutant to plague bacteriophages

We showed previously that the *Y. pestis* EV Δ *lpxM* mutant became resistant to the plague diagnostic bacteriophage L-413C [52], however, it remained sensitive to the Pokrovskaya phage [20]. The analysis of the *Y. pestis* 231 Δ *lpxM* mutant also resulted in the same reactivity, although the parent *Y. pestis* 231 strain was sensitive to both phages. Thus, the mutation in the *lpxM* gene led to the modification of the cell surface of bacteria causing a phage-resistant phenotype which might be an additional indication of the pleiotropic nature of this mutation. Therefore, *Y. pestis* 231 Δ *lpxM* strain displayed L-413C- resistant phenotype similar to that observed for the *Y. pestis* EV NIEG Δ *lpxM* mutant [20].

4. Discussion

The success in development of a new generation of effective plague vaccine generally depends on the existence of a suitable vaccine candidate with desirable characteristics capable of eliciting a marked immunity while having minimal side effects. This can be accomplished by using technologies to significantly improve both the protective and safety characteristics of the vaccine candidate by modifying particular properties to reduce or eliminate the undesirable or harmful effects while maintaining immunogenicity. A live plague vaccine, based on the *Y. pestis* EV line NIEG strain, has been successfully used for prevention of plague infection for over 80 years in the USSR and countries of the FSU [3-6,20] although use outside of this region has been limited by perceived toxicity. In an attempt to reduce toxicity by altering the LPS acylation pattern to one that is less toxic, an *lpxM* mutant of the *Y. pestis* EV line NIEG strain was constructed [20,25] that could only produce a penta-acylated lipid A. This derivative was still avirulent as judged by animal studies (mice, guinea pigs) and provided enhanced protection in animals when compared with the parental strain against experimental bubonic plague caused by the fully virulent wild-

type *Y. pestis* 231 [20]. The *Y. pestis* EV $\Delta lpxM$ bacteria replicated and remained at the site of inoculation significantly longer than the parental EV NIEG strain and did not exhibit any significant transient replication at peripheral sites such as occurs with Δpgm *Y. pestis* strains [4,9,11,15]. However, it was not certain if the observed enhancement in protection provided by the *Y. pestis* EV $\Delta lpxM$ could be attributed solely to decreased LPS toxicity, but might also be due to a pleiotropic phenotype arising from the deletion of the *lpxM* gene leading to the changes in synthesis of other immunoreactive antigens that could either be essential for the development of the immunity to plague or to the re-direction of the way the pathogen interacts with its host [27-32,35,36]. In this report we found multiple changes in the $\Delta lpxM$ *Y. pestis* EV strain indicating that its enhanced virulence and apparently lower toxicity is due to multiple effects resulting from the loss of the ability to synthesise multiple glycoforms of the lipid A.

Within 2-3 hours after inoculation to susceptible mammals with cells of the current *Y. pestis* EV NIEG vaccine, there is decreased production of principal immunoreactive antigens, such as F1, Pla, Ymt and surface-specific polysaccharides (SSP), leading to the reduction of the host innate immune response that normally controls the invading pathogen during first one-three days after vaccination [9,12,39] and thus allowing for sufficient bacterial growth and persistence to activate acquired immune responses. This persistence, together with the well-established modulation of innate immunity by the action of type 3 secretion system (T3SS) [66] could provide a live plague vaccine a beneficial delay in host inflammatory immune responses and thus might allow for a lower dose of vaccine to be used as well as time for cells to reach the regional lymph nodes to initiate acquired immune responses. With the current live vaccine strain, the signs of inflammation in the regional lymph nodes and spleen are not detected earlier than the third to fourth day post-vaccination, and then the inflammation typically disappears by the seventh day after inoculation [9]. During this time there is a toxic effect as a result of intensive bacterial destruction in the regional lymph nodes and spleen tissues [12] as well as progressive tissue hypoxia mediated by damaged erythrocytes [13,42]. Starting at day six to seven, the vaccine strain is killed and eliminated, followed by the appearance of specific serum antibodies to the *Y. pestis* F1 antigen in low titers (1:4 – 1:40) as detected by passive haemagglutination. The emergence in the regional lymph nodes of single B-cells producing specific immunoglobulins to F1 and the ECA of *Y. pestis* has also been reported [9,13]. No live bacterial cells are typically found in the immunised organism by day 13 post-vaccination [9]. This transient residence of the bacterial cells of the live plague vaccine is thought to underlie the emergence of undesirable side effects, although, in general, the toxic effects still quite short and the vaccine does elicit a long-lasting and robust immune response.

We attempted to determine a likely cellular basis for the reduced toxicity and enhanced immunogenicity of the *lpxM*-mutant of *Y. pestis* EV NIEG. The LPS prepared from this strain, as well as the $\Delta lpxM$ -mutant of strain 231, retained a potent adjuvant activity, yet possessed an impaired ability to induce production of TNF- α after stimulation of the macrophage-like cell line J774.A1 *in vitro* (Fig. 5). Moreover, the *Y. pestis* EV $\Delta lpxM$ bacteria had a reduced growth rate and demonstrated an altered resistance to the plague diagnostic bacteriophage L-413C [20,38,52]. We also found changes in production of the major immunoreactive antigens of *Y. pestis* that are involved in an extracellular resistance to phagocytosis, i.e., F1, Ymt, Pla [39] as well as in the V antigen that possesses known immunogenic and immunomodulatory activities [66,67]. There were both quantitative and qualitative changes in the expression of some of these factors, as fine epitope specificity, as revealed by reactivity with MAbs, was also altered for some of these proteins. We found similar changes in epitope specificity on carbohydrate antigens produced by the *lpxM* mutant bacteria. These data provide convincing evidence that the mutation in the *lpxM* gene of the EV NIEG strain likely results in multiple modifications of the structures on the

bacterial surface that appear to be beneficial for the protective potency of a live plague vaccine.

A likely indirect effect of the deletion of the *lpxM* gene could be seen when the production of capsule substance was compared between the mutant and parent strains. The capsule of *Y. pestis* is involved in formation of resistance to phagocytosis [39,56,57] and capsular subunit Caf1 encoded by the large plasmid pMT1 is an established protective antigen [8]. The production of capsule by *Y. pestis* is typically observed at 37 °C, and using a few different methods, we clearly demonstrated that the synthesis of Caf1 by the *lpxM* mutants was significantly reduced. Nonetheless this resulted in a better overall antibody titer to the Caf1 antigen, perhaps due to more optimal dose-effect phenomenon for the induction of the humoral immune response.

The assignment of specific changes in the vaccine properties of the *Y. pestis* EV Δ *lpxM* vaccine strain to alterations in other factors cannot be made. There were reductions or complete loss of a 14-kDa lipoprotein which has been reported to be involved in cytotoxic and hemolytic activities [58], and reduced synthesis at 37 °C of a 28-kDa and a 70-kDa immunoreactive protein of unknown function.

The activity, but not the production, of the plague surface protease Pla was generally decreased in the *lpxM* mutant of EV NIEG, although we failed to detect a similar reduction in the Δ *lpxM* 231 derivative. Strain EV Δ *lpxM* possessed a reduced fibrinolytic activity and an altered rate of conversion of the α -Pla to β -Pla isoform, resulting in the accumulation of the former on the bacterial cell surface. The immunoreactivity of the Pla from the mutant cells with the MAb-Pla was significantly weaker, indicating a change in some of the epitopes on Pla. Notably, the enzymatic activity of the Pla directly correlates with the presence of rough LPS in *Y. pestis* [59,70] which is mainly in the tetra-acylated lipid A glycoform when the bacteria are grown at 37 °C but hexa-acylated in cells grown at 26 °C [20-23,25]. Results with the *Y. pestis* EV Δ *lpxM* strain indicate that the expression of Pla in a penta-acylated LPS environment can influence the functional characteristics of Pla, at least in some strains of *Y. pestis*. The consequence of this decreased Pla activity can be an impaired dissemination of *Y. pestis* from the site of inoculation of the vaccine as well as a change in innate immune response in the regional lymph nodes after subcutaneous immunisation [39,62,69]. The latter can act in a concert with an altered activity of the V antigen, a vigorous immunomodulating factor of *Y. pestis* [66,67], whose production was also reduced in the *lpxM* mutants. Thus, the changes in the activities of Pla and V antigen observed in the *Y. pestis* EV Δ *lpxM* mutant may contribute to an altered innate immune response that can lead to the changes in adaptive immune response to this vaccine in comparison with the parental EV NIEG strain.

Recently, a construction of *Y. pestis* mutant in the *lpp* gene encoding Braun lipoprotein has been reported which displayed an impaired ability to disseminate to the bloodstream and spleens of infected mice [71]. Lpp signals through Toll-like receptor 2 (TLR-2), not TLR-4 like LPS, stimulating the production of proinflammatory cytokines and hence synergising with LPS in induction of septic shock [72]. Thus, double Δ *lpxM* Δ *lpp* mutant might be an excellent candidate to investigate as a target for improved live plague vaccine.

Overall, the advantageous features of the *Y. pestis* EV Δ *lpxM* derivative makes it a potential candidate for an improved live plague vaccine because it can survive longer in the host and is less toxic. The pleiotropic effects of the mutation make it difficult to give a good correspondence among the mutation, the effects on other factors and the properties of the vaccine. Likely the changes collectively contribute to the enhanced vaccine properties of *Y. pestis* EV Δ *lpxM* and given the stability of this change in the strains background provide a

high level of assurance that it will remain safe for human use and has little to no chance of reverting to a more virulent phenotype. The further work is required to study in details the interaction of the *lpxM* mutants with the target cell in the mammalian macro-organism to understand the mechanism of enhanced immunity induced by the *Y. pestis* EV Δ *lpxM*.

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Fig. 1.
Mean of triplicate determinations of the production of capsular antigen F1 by the *Y. pestis* EV NIEG and 231 strains and their $\Delta lpxM$ derivatives in indirect haemagglutination test.

Fig 2. SDS-PAGE stained with Coomassie Brilliant Blue R-250 of untreated (a) and silver-stained proteinase K-treated whole-cell lysates (b) of the *Y. pestis* EV NIEG (lanes 1, 3) and *Y. pestis* EV $\Delta lpxM$ derivative (lanes 2, 4) grown either at 28 °C (lanes 1, 2) or 37 °C (lanes 3, 4).

Fig 3.
Electron micrographs of *Y. pestis* EV NIIEG (a, b) and *Y. pestis* EV Δ *lpxM* derivative (c, d) grown either at 28 °C (a, c) or 37 °C (b, d).

Fig. 4.

Mean of triplicate determinations of antibody titers to capsular antigen F1 and sonicated extracts of cells of *Y. pestis* EV NIEG grown at 28 °C and 37 °C in mice immunized with live EV NIEG (open bars) or EV $\Delta lpxM$ (filled bars) as determined by ELISA.

Fig. 5.

The ability of different types of LPS to stimulate production of TNF- α subsequent to treatment of macrophage-like cell line J774.A1 with 100 ng of LPS. The open and filled bars correspond to LPS obtained from cells grown at 37 °C and 28 °C, respectively. The LPS preparations were as following: the *E. coli* O55:B5, the *Y. pestis* parental strain KM218, the *Y. pestis* KM218 $\Delta lpxM$ and *F. tularensis* 15/10.

Table 1
Immunoreactivity of the MAbs to different carbohydrate antigens of *Y. pestis* and *Y. pseudotuberculosis* in dot-ELISA

Bacterial strain and antigens	PK treatment	Cultivation temperature, C°	Reaction with MAbs													
			1A ₆	4B ₁₁	5E ₆	3F ₇	1G ₈	2D ₁₁	1F ₈	2E ₄	1B ₇	1C ₄	3C ₇	1D ₁₀	2D ₇	6D ₁₁
<i>Y. pestis</i> EV NIEG	native	28	+	-	+	-	+	+	-	+	-	-	+	+	-	-
		37	+	-	-	-	+	+	-	-	-	-	+	+	+	+
	PK-digested	28	+	-	+	-	+	+	-	+	-	-	+	±	-	-
		37	+	-	-	-	+	+	+	-	-	-	+	+	+	+
<i>Y. pestis</i> EV <i>AlpxM</i>	native	28	+	-	+	+	-	+	+	+	+	+	-	-	-	-
		37	+	-	-	-	-	-	-	+	-	-	-	-	-	-
	PK-digested	28	+	-	+	+	-	+	+	+	+	+	-	-	-	-
		37	+	-	-	-	-	-	-	+	-	-	-	-	-	-
<i>Y. pseudotuberculosis</i>	native	28	-	+	+	+	-	-	-	+	+	-	-	-	-	-
		37	-	+	-	-	-	-	-	-	-	-	-	-	-	-
	7 ₁ (I)	28	-	+	+	+	-	-	-	-	+	+	-	-	-	-
		37	-	+	-	-	-	-	-	-	-	-	-	-	-	-
LPS (<i>Y. pestis</i> EV NIEG)		28 ^a	+	-	-	-	-	-	-	-	-	-	-	-	-	-
ECA-30 (<i>Y. pestis</i> EV NIEG)		28 ^a	-	-	-	-	-	-	-	-	-	-	+	+	+	+
ECA-31 (<i>Y. pestis</i> EV NIEG)		37 ^a	-	-	-	-	-	-	-	-	-	-	+	+	+	+
Galactolipid (<i>Y. pestis</i> EV NIEG)		37 ^a	-	-	-	-	+	+	+	+	+	+	-	-	+	+
LPS (<i>Y. pseudotuberculosis</i> , I)		28 ^a	-	+	+	+	-	-	-	-	-	-	-	-	-	-

^aThe cultivation temperature of the bacterial cells used for isolation of the antigen.

Comparison of the ability of the *Y. pestis* EV NIIEG and 231 strains and their *lpxM* derivatives to react with monoclonal and polyclonal antibodies in dot-ELISA

Table 2

<i>Y. pestis</i> strain	Cultivation temperature, C°	Reaction ^a with			
		MAb-F1	MAb-Pla	PFA	MAb-V
EV NIIEG	28	+	++	++	N/D ^b
	37	+++	+++	+++	+++
EV Δ <i>lpxM</i>	28	-	+	+++	N/D ^b
	37	-	+	+++	++
231	28	+	++	++	N/D ^b
	37	+++	+++	+++	+++
231 Δ <i>lpxM</i>	28	+++	+++	+++	N/D ^b
	37	+	++++	++	++

^aPositive (+) or negative (-) reaction. Intensity of staining (color signal) was estimated visually as dark-brown (++++), brown (+++), light brown (++), or trace of color (+)
^bN/D – no data/not determined.

Table 3

Comparative Pla (fibrinolytic and coagulase) activity in the *Y. pestis* EV NIEG and 231 strains and their *lpxM* derivatives

<i>Y. pestis</i> strain	Fibrinolytic activity ^a , hours at 37°C				Coagulase activity ^a , hours at 26°C			
	1	2	4	20	1	2	4	20
EV NIEG	+++	+++	+++	-	-	-	++	+++
EV $\Delta lpxM$	++	+++	+++	+	-	-	++	+++
231	+	+++	+++	++	-	-	+++	+++
231 $\Delta lpxM$	+	+++	+++	++	-	-	+++	+++
None	+++	+++	+++	+++	-	-	-	-

^a Clot occupied all of the volume of the tube (++++), 2/3 of the volume of the tube (+++) or 1/3 of the volume of the tube (++) Trace of a clot (+) or no clot (-).