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Virulence of pPst$^+$ and pPst$^-$ strains of *Yersinia pestis* for guinea-pigs

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Guinea-pigs were infected subcutaneously or by respiratory challenge with plasmid-containing (pPst$^+$pCad$^+$pFra$^+$) *Yersinia pestis* strain 358 and its pPstp$^-$Cad$^+$pFra$^+$, pPst$^+$pCad$^+$pFra$^-$ and pPst$^-$pCad$^+$pFra$^-$ derivatives, grown *in vitro* at 28°C or at 37°C. Lack of plasmid pPst did not lead to an increase in LD50 with either route of challenge. When the virulence of the four *Y. pestis* strains grown at the two temperatures was compared, the LD50 values of those grown at 37°C were lower. Respiratory challenge with cultures grown at 37°C mimics the man-to-man pneumonic plague cycle. The average LD50 values decreased c. two-fold and 10-fold for pPst$^+$ and pPst$^-$ *Y. pestis* variants, respectively. The data suggest that historical epidemic outbreaks of pneumonic plague in the human population residing in the Caucasus region where there are natural plague foci in common voles may have been caused by pPst$^-$ *Y. pestis* strains.

Introduction

The 9.5-kb plasmid (pPst) of *Yersinia pestis* determines plasminogen activator (fibrinolysin), coagulase, pesticin and pesticin immunity activities [1-3], and is necessary for effective invasiveness of the plague pathogen [4-7]. However, it has been shown that *Y. pestis* strains isolated from common voles *(Microtus arvalis* Pall.) obtained in the Caucasus region lack this plasmid, but possess two other plasmids typical of *Y. pestis,* pCad (c. 70 kb) and pFra (c. 100 kb) [8,9]. Epidemics of pneumonic plague in the human population were recorded just in the territory of the natural plague foci in these voles up to the end of the 19th century [10]. Thus, it is possible that during such human plague outbreaks, vole strains (pPst$^-$) of *Y. pestis* were transmitted from man to man, by aerosols, without any flea vector. This type of plague cycle results when a primary case of bubonic plague develops secondary pneumonic plague and infects contacts *via* the respiratory route [11]. It should be noted that marked differences in the phenotype properties of *Y. pestis,* mediated by global regulatory mechanisms, occur with an increase from flea (20-30°C) to host (37-42°C) temperature. These changes include additional nutritional requirements and production of virulence functions [12]. *Y. pestis* cells become highly resistant to mammalian defence mechanisms after growth *in vitro* or *in vivo* for 9–16 h at 37°C [13]. Such cultures grown at 37°C begin multiplication and dissemination in the new host at once, without a preliminary lag phase, and are highly virulent [14].

Another plasmid resident in *Y, pestis,* pFra, encodes the synthesis of capsular antigen ('fraction I', FI), and 'murine' toxin ('fraction II', FII) [15]. It is commonly thought that FI antigen and 'murine' exotoxin serve to enhance the acute form of infection [12]. However, a preliminary study showed that the loss of pFra did not lead to increased LD50 values, and did not influence the survival time of infected animals (mice and guinea-pigs) after subcutaneous challenge. The results of pFra$^-$ strain production under non-selective conditions suggested that such *Y. pestis* variants may arise in natural plague foci at high frequency and may participate in supporting the epizootic process [16].

In this study an attempt was made to mimic the direct interhuman transmission of pPst$^+$ or pPst$^-$ *Y. pestis* strains and their pFra$^-$ derivatives in an aerosol infection model of guinea-pigs.

Materials and methods

Bacterial strains

Y. pestis strains used in the present study are listed in Table 1. Their phenotypes were characterised as

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Table 1. *Y. pestis* strains: relevant attributes

Strain no.	Virulence determinants (phenotype)*	Source or reference
358	$Fra^+Tox^+Lcr^+V^+Pst^+Fb^+Cg^+Pgm^+$	CCIM
358pPst$^-$	$Fra^+Tox^+Lcr^+V^+Pst^-Fb^-Cg^-Pgm^+$	The authors' collection
358pFra$^-$	$Fra^-Tox^-Lcr^+V^+Pst^+Fb^+Cg^+Pgm^+$	Ref. [16]
358pFra$^-$pPst$^-$	$Fra^-Tox^-Lcr^+V^+Pst^-Fb^-Cg^-Pgm^+$	The authors' collection

*Fra, synthesis of capsular antigen fraction I; Tox, synthesis of murine exotoxin; Lcr, low calcium response; V, synthesis of V antigen; Pst, synthesis of pesticin; Fb, fibrinolysin activity; Cg, coagulase activity; Pgm, pigmentation. CCIM, The Culture Collection of Institute "Microbe".

described previously [17]. The parent wild-type strain 358 was isolated from a fatal human case of bubonoseptic plague in Kazakhstan by A. Kh. Arslanova and A. F. Kalmykova in 1955. Incubation of *Y. pestis* cultures on agar medium at 4°C enhanced the frequency of pPst elimination [18]. The pFra$^-$ derivatives used were generated as described previously [16].

Media and culture conditions

The cultures were grown at 28°C and at 37°C on Luria Bertani Medium (LB; containing/L,: tryptone 10 g, yeast extract 5 g, NaCl 10 g) supplemented with agar 2%, pH 7.2.

Plasmid content

Plasmids were screened by the method of Birnboim and Doly [19]. The plasmid profile data (not shown) were confirmed by PCR amplification of plasmid-encoded gene (*pla*sminogen activator, *pla,* and *c*apsular *a*ntigen *f*raction *I*, *caf1*) fragments as described previously [20].

Virulence for guinea-pigs

Y. pestis cultures grown at 28°C were used to model two types of plague cycle: (i) rodents/rodent fleas/ man-bubonic plague (subcutaneous route), and (ii) rodents/contaminated soil (dust)/man-pneumonic plague (respiratory route). Cultures grown at 37°C were used in simulating the man-to-man—pneumonic plague cycle (respiratory route).

Bacteria grown for 48 h at 28°C on LB agar were suspended in saline (NaCl 0.89% w/v) to 5×10^2 cfu/ml; 0.2-ml volumes of 10-fold dilutions were injected subcutaneously into outbred female guinea-pigs weighing c. 250 g.

Other groups of guinea-pigs were infected by respiretory challenge in the aerodynamic chamber designed in the Institute 'Microbe'. Bacteria grown at 28°C or at 37°C for 48 h were suspended to 10^7 cfu/ml in aqueous lactose 10% w/v. Different groups of animals were exposed for different times (2, 4, 8 and 16 min). Six animals were challenged with each aspiration dose and spraying was with a uniflow pneumatic sprayer of the ejection type. It created an aerosol consisting mainly (90.3%) of particles < 2 μm in diameter. The aerosol particles were sized as described previously [21]. After the challenge procedure, aerosol samples were collected by the aspiration-filtration method with impingers filled with saline. By the end of the challenge procedure, biological aerosol concentration (AC), i.e., number of cfu/L of the chamber volume, was determined by quantitative culture. The aspiration dose (AD) received by the guinea-pig in the chamber was calculated from the formula:

$$AD = AC \times V \times t$$

in which V, the volume of air inspired/min/guinea-pig, was taken as 0.1 L and t was the challenge time.

Animals that succumbed to infection were dissected and examined bacteriologically. The remaining animals were observed for 21 days. LD50 values and 95% confidence intervals were determined according to the method of Kärber, as modified by Ashmarin and Vorob'ov [22].

Results

Comparative analyses of Y. pestis pPst$^+$ and pPst$^-$ strains

There were no significant differences in LD50 values between pPst$^+$ strains and their pPst$^-$ derivatives in each of the three groups of guinea-pigs: (i) infected subcutaneously with cultures grown at 28°C; (ii) infected by aerosol challenge with cultures grown at 28°C; and (iii) infected by aerosol challenge with cultures grown at 37°C (Fig. la and c). When the virulence of the four *Y. pestis* strains grown at the different temperatures was compared, the LD50 values of those grown at 37°C were lower. These differences, taking into account the confidence intervals, were significant only for the two pPst$^-$ strains. Average LD50 values decreased *c* two-fold and 10-fold for the pPst$^+$ and pPst$^-$ *Y. pestis* variants, respectively.

Analysis of average survival time of infected animals revealed two tendencies, (i) survival time in the guinea-pigs challenged subcutaneously with cultures of pPst$^+$ *Y. pestis* grown at 28°C changed in the case of pPst loss (the differences were significant) in the opposite direction to that in animals exposed to

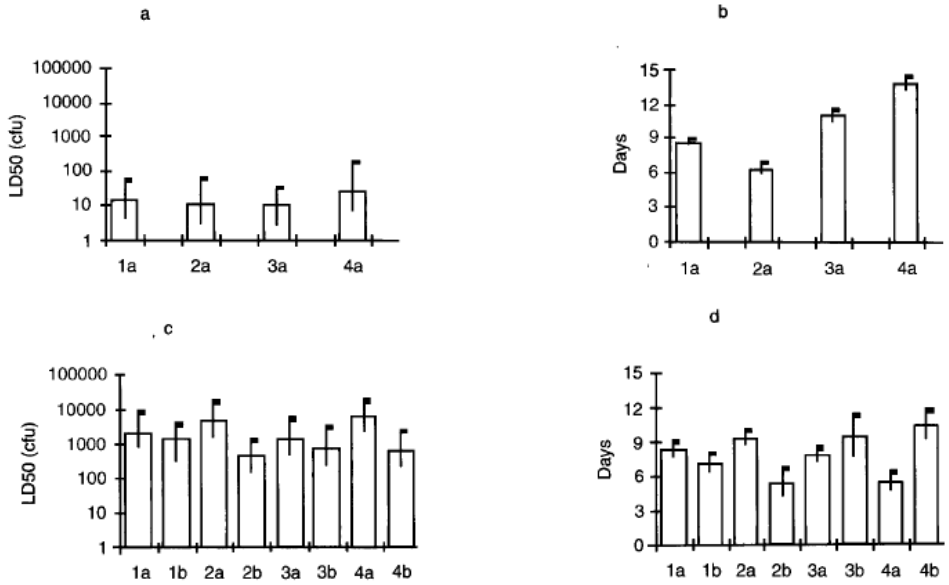

Fig. 1. LD50 (a, c) and average survival time (b, d) in guinea-pigs infected subcutaneously (a, b) or by respiratory challenge (c, d) with $pPst^-$ strains and their parent variants cultivated at 28°C (a) or 37°C (b). 1, strain no. 358; 2, 358$pPst^-$; 3, 358$pFra^-$; 4, 358$pFra^-pPst^-$. Bars represent 95% confidence intervals.

respiratory challenge with cultures grown at 28°C (the differences were significant for $pFra^-$ strains) (compare 1a, 2a, 3a and 4a in Fig. 1b and d), but correlated with the direction of changes when cultures grown at 37°C were used (the differences were significant for $pFra^+$ strains) (compare 1a, 2a, 3a and 4a in Fig. 1b and 1b, 2b, 3b and 4b in Fig. 1d). (ii) Presence of pFra led to a decrease of survival time in animals infected by respiratory challenge when cultures of *Y. pestis* grown at 37°C were compared with those grown at 28°C (compare 1a, 1b, 2a and 2b in Fig. 1d). Conversely, lack of pFra led to its increase (compare 3a, 3b, 4a and 4b in Fig. 1d); the differences were significant for $pPst^-$ strains.

With each of the three groups of infected guinea-pigs, *Y. pestis* was isolated from regional lymph nodes, liver and spleen from every animal that died after inoculation. None of the uninoculated guinea-pigs, which were held in separate cages in the same room as the infected guinea-pigs, died. The deaths associated with *Y. pestis* inoculations were, therefore, assumed to be due to plague.

Discussion

It is known that both plasminogen activator and coagulase are determined by the same gene - designated *pla,* [23] encoded by plasmid pPst - which encodes surface protease Pla [7,24]. The *pla* gene has been reported to be required for high virulence in cases of subcutaneous challenge of mice and guinea-pigs [4, 5,7] and alimentary challenge of mice [25]. However, according to the other data, loss of plasmid pPst did not lead to decrease in virulence for mice and guinea-pigs after subcutaneous challenge [16,25,26]. However, microbial pathogenesis is usually complex and mutifactorial. Several virulence factors may act individually or in concert to produce infection. Removal of any one of these components may or may not render the organism avirulent [27]. Differences in experimental data may be due to the use of different parent strains that could possess unidentified mutations decreasing the potential store of *Y. pestis* virulence factors. Elimination of one more factor from such potentially weakened strains could lead to a significant decrease in virulence. The present data confirm this assumption. Simultaneous loss of both pFra and pPst, but not pPst only, resulted in increased survival time in guinea-pigs challenged subcutaneously. In our opinion, the use of such potentially weakened variants of bacteria may be useful for more effective analysis of virulence determinants.

It has been suggested recently that pPst plasmid acquisition [7], but not gene-inactivating mutations [28], was a major event in the course of plague pathogen evolution from *Y. pseudotuberculosis.* The existence in nature of vole strains of *Y. pestis* lacking pPst contradicts this hypothesis.

The most dangerous of externally disseminating forms of black death, initial-pulmonary and initial-intestinal plague, are characterised by colonisation of the host mucosal surfaces [29], where IgA is the predominant

secretory antibody isotype [30]. It is known that some bacterial species produce IgA proteases which presumably enhances their ability to survive at mucosal surfaces [27, 30-33]. We can speculate *a priori* that *Y. pestis* surface protease Pla may also hydrolyse IgA. Furthermore, pPst plasmid encodes the bacteriocin pesticin [1-3], providing *Y. pestis* with the ability to suppress other pathogenic yersiniae [12] that possess similar mechanisms of inorganic iron uptake [34]. Moreover, it has been shown that *Y. pestis* wild-type strains are more virulent than their isogenic pPst$^-$ derivatives *via* the alimentary route. Alimentary challenge was performed by feeding the experimental animals with mice that had died after subcutaneous challenge (simulation of cannibalism). Challenge with the wild-type strain resulted in the deaths of 71.2% of the animals. The variants lacking pFra, pPst and the two plasmids simultaneously caused a mortality of 54.1%, 20% and 15.6%, respectively [25].

The observation that, in some cases, pPst$^-$ strains caused a reduced survival time in infected animals seemed to be surprising. However, the data in Fig. 1d (1a, 1b, 2a, 2b) suggest that the pPst products are advantageous to *Y. pestis* grown at 28°C. These products may enable the organism to overcome host pulmonary defences for a period of a time to allow expression of virulence factors which, in turn, enable it to persist and become lethal at the host temperature (37°C). *Y. pestis* cultures grown at 37°C, in contrast to those grown at 28°C, can survive and even grow significantly within phagocytes [13] and, on entering pulmonary macrophages, at once begin rapid multiplication and acquire the ability to resist clearance from the lungs of guinea-pigs [35]. In this case, Pla invasin is no longer necessary for dissemination of bacteria. Thus, diversion of energy sources of the microbial cell away from synthesis of the plasmid pPst products to the synthesis of other virulence factors and to cell division of *Y. pestis* pPst$^-$ variants may lead to decreased survival time of the host. Capsular antigen FI is believed to confer resistance to phagocytosis. The additional loss of ability of FI synthesis (Fig. 1d; 3a, 3b, 4a, 4b) resulted in increased duration of survival of the infected animals.

Several properties have been suggested for the *pla* gene and Pla protein (i) The alternative forms of the Pla protein are essential to 'flea blockage' and subsequent transmission of the plague bacillus to animals [36]; (ii) expression of the *pla* gene product may contribute to the deleterious effects of *Y. pestis* on fleas [37]; (iii) when the cloned *pla* locus was introduced into *Escherichia coli,* it conferred upon the bacterium the ability to bind to a number of cell lines [38]; and (iv) the Pla protease was shown to cleave complement C3 at a specific site [7]. Our opinion is that the main but not the only role of the pPst plasmid in the evolution of the plague pathogen is to provide effective mechanisms of competition for an ecological niche, the mammalian intestine, between *Y. pestis* and related *Yersinia* species. As we see it, the 9.5-kb plasmid products in *Y. pestis* are necessary to implement the oral route of transmission (by cannibalism or by the feeding of fleas). It is hoped that further work in this area will clarify the mechanisms by which *pla* acts and its roles in the pathogenesis of plague.

Finally, the data concerning respiratory challenge of guinea-pigs and the finding that *Y. pestis* strains lacking plasmid pPst were as virulent as the wild-type strains support the concept that historical epidemic outbreaks of pneumonic plague in the human population residing in the Caucasus region, where there are natural plague foci in common voles, were caused by these pPst$^-$ strains.

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