The subcutaneous inoculation of pH 6 antigen mutants of *Yersinia pestis* does not affect virulence and immune response in mice

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Two isogenic sets of *Yersinia pestis* strains were generated, composed of wild-type strains 231 and I-1996, their non-polar pH 6[−] mutants with deletions in the *psaA* gene that codes for its structural subunit or the whole operon, as well as strains with restored ability for temperature- and pH-dependent synthesis of adhesion pili or constitutive production of pH 6 antigen. The mutants were generated by site-directed mutagenesis of the *psa* operon and subsequent complementation *in trans*. It was shown that the loss of synthesis or constitutive production of pH 6 antigen did not influence *Y. pestis* virulence or the average survival time of subcutaneously inoculated BALB/c naïve mice or animals immunized with this antigen.

INTRODUCTION

Studying mechanisms of host–pathogen interactions is necessary in the search for novel potential molecular targets to develop effective modern methods for laboratory diagnosis, vaccine prophylaxis and therapy (Li *et al.*, 2007; Marra, 2004). The plague pathogen, *Yersinia pestis*, offers a classical model for the study of interactions of bacterial pathogens with the host organism (Anisimov, 2002a, b; Brubaker, 2006; Burrows, 1957, 1963; Jarrett *et al.*, 2004; Lorange *et al.*, 2005; Perry & Fetherston, 1997). The three plague pandemics killed more than 200 million humans. This disease is a typical zoonosis that circulates within natural plague foci in populations of more than 200 rodent and lagomorph species (Anisimov *et al.*, 2004; Gage

& Kosoy, 2005). Plague is transmitted by at least 80 species of fleas (Anisimov *et al.*, 2004; Gage & Kosoy, 2005). As a rule, morbidity in humans is found when epizootics become acute and it is a consequence of bites from infected fleas or direct contact with infected animal tissues (Anisimov *et al.*, 2004; Gage & Kosoy, 2005; Perry & Fetherston, 1997).

The extraordinarily high virulence of *Y. pestis* (Lorange *et al.*, 2005) is determined by a wide spectrum of its features, initially termed as ‘determinants of virulence’ (Burrows, 1957, 1963). The ‘classic’ determinants include the ability of bacterial cells to absorb exogenous dyes and haemin (Pgm⁺), dependence on growth at 37 °C using Ca²⁺ ions present in the medium (Ca⁺), synthesis of V and W antigens, synthesis of ‘murine’ toxin and capsule antigen F1 (Tox⁺ and Fra⁺), synthesis of pesticin concomitant with fibrinolytic and coagulase activities (Pst⁺, Fb⁺ and Cg⁺), purine independence or the ability to produce endogenous purines (Pur⁺). After five decades of research and deliberation stemming from the initial proposal of ‘virulence determinants’, some of them, such as W antigen, pesticin and the ability to produce endogenous purines, are no longer recognized as pathogenicity factors (Anisimov, 2002a, b; Brubaker, 2006; Perry & Fetherston, 1997). Recently, a number of additional virulence factors have

Abbreviations: Ap^R, ampicillin-resistant; Ap^S, ampicillin-sensitive; BHI, brain heart infusion; Cm^R, chloramphenicol-resistant; i.p., intraperitoneal; i.v., intravenous; Km^R, kanamycin-resistant; Pm^R, polymyxin B-resistant; s.c., subcutaneous; Suc^R, sucrose-resistant; Tc^R, tetracycline-resistant; TSISCBP, Tarasevich State Institute for Standardization and Control of Biomedical Preparations.

The GenBank/EMBL/DDBJ accession number for the determined nucleotide sequence of the *psa* operon of *Y. pestis* strain 231 is EF079883.

A supplementary table of primer sequences is available with the online version of this paper.

been identified. They include secreted *Yersinia* outer proteins (Yops) (Cornelis, 2002), adhesion pili (pH 6 antigen, termed PsaA) (Lindler & Tall, 1993; Vodop'ianov & Mishan'kin, 1985; Vodop'ianov *et al.*, 1990) and six additional chaperone/usher systems (Felek *et al.*, 2007): YadA-like non-pilus adhesin encoded by bicistronic operon *yadBC* (Forman *et al.*, 2008), lipopolysaccharide (Bengoechea *et al.*, 1998; Knirel *et al.*, 2007; Oyston *et al.*, 2003; Porat *et al.*, 1995), murein (or Braun) lipoprotein Lpp (Sha *et al.*, 2008) and others (Anisimov, 2002a, b; Brubaker, 2006; Perry & Fetherston, 1997).

pH 6 antigen was initially described in 1961 as an antigen synthesized by *Y. pestis* at the temperature close to body temperature of mammals (35–41 °C) and acidic pH values (5.8–6.0) close to the pH of abscesses or phagolysosomes in macrophages (Ben-Efraim *et al.*, 1961). Recently, it was shown that growth in human plasma also upregulated transcription of *psaE* and *psaF*, suggesting a role for pH 6 antigen in the septicemic phase of plague (Chauvaux *et al.*, 2007). More recently, gene expression studies demonstrated highly persistent expression of the *psaA* gene from 4 to 48 h after intranasal infection in mouse lungs compared with the spleen and liver, indicating possible involvement of this antigen in the pathogenesis of pneumonic plague (Liu *et al.*, in press). Subunits of pH 6 antigen, which have a molecular mass of 15 kDa, assemble on the surface of *Y. pestis* and *Yersinia pseudotuberculosis* into homopolymer macromolecular complexes termed adhesion pili (Lindler & Tall, 1993; Vodop'ianov & Mishan'kin, 1985; Vodop'ianov *et al.*, 1990), forming together a capsule-like structure similar to that formed by *Y. pestis* F1 capsular antigen (Anisimov, 1999; Cherepanov *et al.*, 1998).

In addition, pH 6 antigen possesses adhesion activity toward erythrocytes (Ben-Efraim *et al.*, 1961; Bichowsky-Slomnicki & Ben-Efraim, 1963; Stepanshina *et al.*, 1993) and mammalian epithelial cells (Galván *et al.*, 2007; Isberg, 1989a, b; Liu *et al.*, 2006; Yang *et al.*, 1996) due to the binding of phosphatidylcholine (Galván *et al.*, 2007) or glycosphingolipids (Payne *et al.*, 1998). It is known to prevent phagocytosis (Huang & Lindler, 2004; Stepanshina *et al.*, 1993), probably through masking the bacterial surface by binding apolipoprotein B-containing lipoproteins from human plasma (Makoveichuk *et al.*, 2003), phosphatidylcholine of pulmonary surfactant (Galván *et al.*, 2007) and/or human IgG1, IgG2 and IgG3 subclasses (Zav'yalov *et al.*, 1996) or other extracellular matrix components, including fibronectin and mucin (Vodop'ianov *et al.*, 1993). In early studies, pH 6 antigen was shown to be cytotoxic for peritoneal (Bichowsky-Slomnicki & Ben-Efraim, 1963) and alveolar (Stepanshina *et al.*, 1993) macrophages. However, high-purity preparations of pH 6 antigen were not cytotoxic for eukaryotic cells (Bakhteeva *et al.*, 2007). Subcutaneous (s.c.) injection of pH 6 antigen to rabbits (1000 µg), guinea pigs (625 µg) and mice (100 µg) did not lead to death (Stepanshina *et al.*, 1993).

The synthesis of pH 6 antigen is encoded by the *psa* operon which has a structure similar to other operons coding for pilus adhesins that are conveyed to the bacterial surface by the chaperone/usher secretion systems (Felek *et al.*, 2007; Hultgren *et al.*, 1993; Iriarte & Cornelis, 1995; Karlyshev *et al.*, 1994; Thanassi *et al.*, 1998). The *psa* operon consists of *psaE* and *psaF*, two regulatory genes responsible for temperature- (37 °C) and pH- (5.8–6.0) dependent transcription regulation, *psaA*, the structural gene of pH 6 antigen subunit, and *psaB* and *psaC*, coding for periplasmic chaperone and molecular usher, respectively (Lindler *et al.*, 1990; Yang *et al.*, 1996). Expression of the *psa* operon is positively regulated by binding of the global transcription factor RovA to the *psaE* and *psaA* promoter regions (Cathelyn *et al.*, 2006), while Fur protein acts as a repressor of *psa* genes (Zhou *et al.*, 2006). A Δ *psaA* mutant of *Y. pestis* wild-type strain CO92 had a significant dissemination defect after s.c. infection, but only slight attenuation by the pneumonic disease model (Cathelyn *et al.*, 2006). Mutations in *psaE* or *psaA* of attenuated Pgm[−] *Y. pestis* strain KIM5 caused a 200-fold reduction of virulence in retroorbitally infected mice (Lindler *et al.*, 1990), while *psaF* mutants of wild-type strain 231 were completely avirulent for mice and guinea pigs after s.c. challenge (Panfertsev *et al.*, 1991). These reports suggest that further studies involving the use of mutants with complete loss of the *psa* operon are required to clarify the role of the pH 6 antigen in the virulence of *Y. pestis*.

The contradictory data on the role of pH 6 antigen in *Y. pestis* virulence might be a result of different strain backgrounds and/or methodological differences in the generation of such strains. It was previously suggested (Domaradskii, 1987) that 'transfers of genetic information are more frequent in the case of atypical forms of certain bacteria. In the case of such an explanation, one should speak not of a decrease in virulence of the cells under the influence of plasmids but of a change in the composition of the population, caused by the accumulation (selection) of recombinants with an initially low virulence or entirely avirulent'. The selection of clones that have retained virulence at the level of the wild-type parent strains requires an animal passage. In evaluating the virulence of cultures of agents of infectious diseases subjected to experimental manipulations or stored for long periods under laboratory conditions, a preliminary stage of animal passage is necessary to purify the microbial population from avirulent segregants (in the case of *Y. pestis*, these are bacteria with the phenotypes Lcr[−], Pgm[−], etc.) (Anisimov, 1999).

In this study, we generated Δ *psaA* and Δ *psaEFABC* mutants that were deficient in the ability to produce pH 6 antigen. We performed their complementation *in trans* using recombinant plasmids coding for the complete cluster of *psa* genes or the *psaABC* locus. Recombinant bacteria were passaged through mice and the effect of s.c. inoculation on the virulence of isogenic *Y. pestis* strains in both naïve and pH 6 antigen-immunized mice was determined.

METHODS

Bacterial strains. *Y. pestis* strain 231 and its derivative KM218 were obtained from the Russian Research Anti-Plague Institute 'microbe' (Saratov, Russia), *Y. pestis* strain I-1996 was obtained from the Anti-Plague Research Institute of Siberia and Far East (Irkutsk, Russia) and *Y. pestis* vaccine strain EV line NIEG was from the Tarasevich State Institute for Standardization and Control of Biomedical Preparations (TSISCBP) (Moscow, Russia). Characteristics of the strains are given in Table 1. *Escherichia coli* strain S17-1 λ pir B was used as a donor for conjugative transfer of recombinant plasmids (Simon *et al.*, 1983). Bacterial cultures were started from lyophilized stocks. For animal challenges, *Y. pestis* strains were passaged in mice as described below. All experiments with the virulent *Y. pestis* strains 231 and I-1996 and their derivatives were performed in biosafety level 3 facilities.

Animals. Male and female BALB/c mice (Lab Animals Breeding Center, Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russia) 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, and allowed to feed and drink *ad libitum* throughout the course of this study.

Animal passage. To select for virulent *Y. pestis* subcultures, a group of four mice was challenged subcutaneously with 0.2 ml aliquots of strains 231 and I-1996 or their derivatives at a concentration of approximately 10^8 c.f.u. The animals that were first to succumb to infection were subjected to necropsy and one bacteriologic loop of the brain tissues was suspended in 1 ml 0.9% NaCl. In the second round of animal passage, four mice were challenged subcutaneously with 0.1 ml of this suspension containing approximately 200 c.f.u. *Y. pestis*. The cultures isolated from the animals that succumbed to early infection in the second passage were used in subsequent experiments.

Growth of bacteria. For mutagenesis, complementation and animal challenge experiments, *Y. pestis* strains were grown at 28 °C for 48 h on brain heart infusion (BHI; HiMedia Laboratories) supplemented with 2% agar at pH 7.2. For testing pH 6 antigen production, bacteria were cultured at 37 °C for 48 h on BHI agar at pH 5.8. For isolation of preparative amounts of pH 6 antigen, BHI, pH 5.8, supplemented with 0.5% yeast extract (Difco) (Lindler *et al.*, 1990) was used. BHI agar with 5% sucrose was used for the selection of *Y. pestis* recombinants containing the suicide vector pCVD442 (Donnenberg & Kaper, 1991). *E. coli* strain S17-1 λ pir and its derivatives were grown at 37 °C for 24 h in Luria-Bertani broth, pH 7.2 (Miller, 1972), or on the same medium supplemented with 2% agar. Growth media were supplemented, as needed, with ampicillin ($100 \mu\text{g ml}^{-1}$), kanamycin ($40 \mu\text{g ml}^{-1}$), chloramphenicol ($10 \mu\text{g ml}^{-1}$) or polymyxin B ($25 \mu\text{g ml}^{-1}$).

Modifications of animal passage procedures used for mutant derivatives of strains 231 and I-1996 are shown below.

Mutagenesis. Deletions in the *psa* operon in *Y. pestis* vaccine strain EV were achieved by allelic exchange using a kanamycin resistance (*kan*) cassette with short flanking homologous regions of the *Y. pestis* target DNA (Datsenko & Wanner, 2000). The *kan* cassettes were obtained by PCR amplification using plasmid pUTKm as template and primers PsaAkm1/PsaAkm2 to generate EV Δ psaA and PsaEkm1/PsaCkm2 to generate EV Δ psaEFABC (Supplementary Table S1, available in JMM Online). The PCR products were introduced by electroporation into *Y. pestis* EVpKD46, as described previously (Conchas & Carniel, 1990). Recombinant colonies were selected on kanamycin BHI agar plates. Correct insertion of the antibiotic resistance cassette was verified by PCR and the inability to produce pH 6 antigen.

Deletions in the *psa* operon in *Y. pestis* virulent strains were achieved using the suicide vector pCVD442 (Donnenberg & Kaper, 1991) in order to prevent possible aerosolization of highly virulent *Y. pestis* that may happen during electroporation, which is necessary for the method described above. Mutant alleles of Δ psaA and Δ psaEFABC were amplified from chromosomal DNA of *Y. pestis* strains EV Δ psaA and EV Δ psaEFABC, respectively, with primer pairs PsaAkm3/PsaAkm4 for Δ psaA and PsaEkm3/PsaCkm4 for Δ psaEFABC, and the fragments containing the mutated genes were ligated into the *Sma*I site of suicide vector pCVD442 (Donnenberg & Kaper, 1991). The ligated plasmids were electroporated into *E. coli* S17-1 λ pir cells to form pCVD442 Δ psaA::*kan* and pCVD442 Δ psaEFABC::*kan*, these were introduced into wild-type *Y. pestis* 231 or I-1996 from *E. coli* S17-1 λ pir by conjugation using polymyxin B for counterselection, to produce kanamycin-, ampicillin- and polymyxin B-resistant ($\text{Km}^R\text{Ap}^R\text{Pm}^R$) merodiploid transconjugants. The $\text{Km}^R\text{Ap}^R\text{Pm}^R$ transconjugants selected for each plasmid/strain combination were plated onto BHI agar with 5% sucrose and grown at 28 °C for 2 days. The resultant sucrose-resistant (Suc^R) Km^R ampicillin-sensitive (Ap^S) colonies were screened by using PCR with the corresponding primer pair and by serological screening for the inability to produce pH 6 antigen. $\text{Km}^R\text{Ap}^S\text{pH } 6^-$ *Y. pestis* *psa* double-crossover mutants (Table 1) for each plasmid/strain combination were pooled and 10^6 c.f.u. of each culture were used for intraperitoneal (i.p.) challenge of four mice. The cultures on antibiotic-free BHI agar isolated from spleens of animals that succumbed to infection were used for a second i.p. challenge of four mice (dose 10^4 c.f.u.). The strains isolated from the second round of animal passage were checked for the inability to produce pH 6 antigen and used for further studies.

PCR verification. Three PCRs were used to show that all mutants had the correct structure. The common test primers for *kan* were k1 and k2 (Supplementary Table S1). Two reactions were performed using flanking locus-specific primers with the respective common test primer (k1, k2) to test for new junction fragments. A third reaction was carried out with the flanking locus-specific primers to verify the loss of the parental (non-mutant) fragment and the presence of the new mutant-specific fragment. Control colonies were always tested in parallel.

Complementation of knockout mutants. Complementation was performed with the plasmid pJG428 [Ap^R tetracycline-resistant (Tc^R)] containing the complete operon *psaEFABC* from *Y. pestis* EV (Makoveichuk *et al.*, 2003) cloned in the cosmid pHC79 (Hohn & Collins, 1980). A *Sau*I-*Cl*aI-fragment of the plasmid pJG428 containing locus *psaABC* was ligated into the vector pBluescript II KS(+) (Stratagene), which was previously digested with *Sau*I and *Cl*aI. The resulting construct was digested with *Pvu*II and ligated to an 800 kb fragment from plasmid pBR325 (Prentki *et al.*, 1981) containing the chloramphenicol resistance (*cat*) gene. The ligated plasmid was electroporated into *E. coli* DH5 α cells to form pIG924Cm [chloramphenicol-resistant (Cm^R)].

Plasmids pJG428 and pIG924Cm were introduced into *Y. pestis* mutants by electroporation (Conchas & Carniel, 1990). Transformants of mutant derivatives of *Y. pestis* strains 231 and I-1996 for each plasmid/strain combination were combined and used for the challenge of four mice (10^6 c.f.u. per animal). The bacterial cultures, which were isolated from spleens of animals on nutrient media with appropriate antibiotics, were used for a second round of infection of four new mice (10^4 c.f.u. per animal). The bacterial cultures isolated after the second round of passage were tested clonally for the presence of antibiotic resistance and ability to produce pH 6 antigen. pH 6^+ clones of each strain were pooled and used for virulence testing.

Table 1. Strains and plasmids used and constructed in this study

More detailed information on biovar-subspecies interrelations and geographical locations of the natural plague foci have been discussed previously (Anisimov *et al.*, 2004).

Strain or plasmid	Description	Source or reference
<i>Y. pestis</i> strains		
231	pFra ⁺ pCD ⁺ pPst ⁺ pH 6 ⁺ Pgm ⁺ Pm ^R wild-type virulent (LD ₅₀ for mice <10 c.f.u., LD ₅₀ for guinea pigs <10 c.f.u.) bv. antiqua ssp. <i>pestis</i> strain from Aksai focus (no. 33), Kirghizia	Drozdoz <i>et al.</i> (1995)
231Δ <i>psaEFABC</i>	As 231, but Δ <i>psaEFABC</i> (pH 6 ⁻)	This study
231Δ <i>psaEFABC</i> pJG428	As 231Δ <i>psaEFABC</i> , but harbouring plasmid pJG428 (pH 6 ⁺)	This study
231Δ <i>psaEFABC</i> pIG924Cm	As 231Δ <i>psaEFABC</i> , but harbouring plasmid pIG924Cm (pH 6 ⁺)	This study
I-1996	pFra ⁺ pCD ⁺ pPst ⁺ pH 6 ⁺ Pgm ⁺ Pm ^R wild-type virulent (LD ₅₀ for mice <10 c.f.u., LD ₅₀ for guinea pigs <10 c.f.u.) Russian bv. antiqua ssp. <i>pestis</i> strain from Trans-Baikal focus (no. 38)	Anisimov <i>et al.</i> (2007)
I-1996Δ <i>psaA</i>	As I-1996, but Δ <i>psaA</i> (pH 6 ⁻)	This study
I-1996Δ <i>psaEFABC</i>	As I-1996, but Δ <i>psaEFABC</i> (pH 6 ⁻)	This study
I-1996Δ <i>psaEFABC</i> pIG924Cm	As I-1996Δ <i>psaEFABC</i> , but harbouring plasmid pIG924Cm (pH 6 ⁺)	This study
EV line NIEG	pFra ⁺ pCD ⁺ pPst ⁺ pH 6 ⁺ Pgm ⁻ (Δ <i>pgm</i>) Pm ^R . Russian vaccine bv. orientalis ssp. <i>pestis</i> strain	TSISCBP
EVpKD46	As EV, but harbouring plasmid pKD46	This study
KM218	As EV, but pFra ⁻ pCD ⁻ pPst ⁻	Knirel <i>et al.</i> (2005)
EVΔ <i>psaA</i>	As EVpKD46, but Δ <i>psaA</i> (pH 6 ⁻)	This study
EVΔ <i>psaEFABC</i>	As EVpKD46, but Δ <i>psaEFABC</i> (pH 6 ⁻)	This study
EVΔ <i>psaEFABC</i> pJG428	As EVΔ <i>psaEFABC</i> , but harbouring plasmid pJG428 (pH 6 ⁺)	This study
EVΔ <i>psaEFABC</i> pIG924Cm	As EVΔ <i>psaEFABC</i> , but harbouring plasmid pIG924Cm (pH 6 ⁺)	This study
<i>E. coli</i> strains		
DH5α	<i>lacZ</i> Δ <i>M15</i> Δ(<i>lacZYA-argF</i>) <i>recA1</i> <i>endA1</i> <i>hsdR17</i> (r _K ⁻ m _K ⁺) <i>phoA</i> <i>supE44</i> <i>thi</i> <i>gyrA96</i> <i>relA1</i>	Sambrook <i>et al.</i> (1989)
DH5αpIG924Cm	As DH5α, but harbouring plasmid pIG924Cm (pH 6 ⁺)	This study
S17-1 λ <i>pir</i>	λ <i>pir</i> lysogen of S17-1 (<i>thi pro</i> <i>hsdR</i> ⁻ <i>hsdM</i> ⁺ <i>recA</i> RP4 2-Tc::Mu-Km::Tn7 (Tc ^R Sm ^R Pm ^S)	Simon <i>et al.</i> (1983)
S17-1 λ <i>pir</i> pCVD442Δ <i>psaA</i> :: <i>kan</i>	As S17-1 λ <i>pir</i> , but harbouring plasmid pCVD442Δ <i>psaA</i> :: <i>kan</i>	This study
S17-1 λ <i>pir</i> pCVD442Δ <i>psaEFABC</i> :: <i>kan</i>	As S17-1 λ <i>pir</i> , but harbouring plasmid pCVD442Δ <i>psaEFABC</i> :: <i>kan</i>	This study
Plasmids		
pBR325	Source of Cm ^R cassette, Ap ^R Cm ^R Tc ^R	Prentki <i>et al.</i> (1981)
pKD46	λ Red recombinase expression plasmid, Ap ^R	Datsenko & Wanner (2000)
pUTKm	Source of Km ^R cassette (Tn5 delivery plasmid), Ap ^R Km ^R	Herrero <i>et al.</i> (1990)
pCVD442	Suicide vector, <i>sacB</i> , Ap ^R	Donnenberg & Kaper (1991)
pCVD442Δ <i>psaA</i> :: <i>kan</i>	Δ <i>psaA</i> :: <i>kan</i> locus from EVΔ <i>psaA</i> cloned in pCVD442	This study
pCVD442Δ <i>psaEFABC</i> :: <i>kan</i>	Δ <i>psaEFABC</i> :: <i>kan</i> locus from EVΔ <i>psaEFABC</i> :: <i>kan</i> cloned in pCVD442	This study
pJG428	Complete <i>psaEFABC</i> operon (<i>KpnI</i> - <i>Clal</i> fragment from chromosome of <i>Y. pestis</i> EV) cloned in cosmid pHc79, Ap ^R Tc ^R	This study
pBluescript II KS(+)	f1 Origin in (+) orientation, <i>Kpn</i> → <i>Sac</i> polylinker orientation, Ap ^R	Stratagene
pIG924Cm	<i>psaABC</i> locus cloned in pKSBluescriptII, Cm ^R	This study

Estimation of pH 6 antigen production. pH 6 antigen production was estimated by double diffusion analysis in agar (Öuchterlony, 1949), Western blot (Yang *et al.*, 1996), standard ELISA (Ngo & Leshoff, 1985) and/or dot-ELISA (Chandrika *et al.*, 1998), using rabbit antiserum directed against *Y. pestis* pH 6 antigen (kindly provided by T. B. Kravchenko).

Isolation and purification of pH 6 antigen. Highly purified pH 6 antigen was prepared by the ammonium sulfate method

(Makoveichuk *et al.*, 2003) from culture supernatants of *Y. pestis* strain KM218 after incubation at pH 5.8 at 37 °C for 48 h.

Animal immunization and virulence challenge. Ninety-six of 224 mice were immunized with pH 6 antigen diluted in 0.1 ml PBS (pH 7.2), containing 10 µg pH 6 antigen, which was administered at a single s.c. site on the back of the animals. After 21 days, the animals were boosted with an identical dose at the same inoculation site. pH 6 antigen antibody titres were measured 26 days after the second pH 6

antigen immunization in four mice by ELISA, using 200 ng of *Y. pestis*-derived pH 6 antigen. The animals were anaesthetized by carbonic gas inhalation prior to the collection of a terminal blood sample by cardiac puncture. Twenty-eight days after the pH 6 antigen booster dose, six immunized and eight naïve animal groups were subcutaneously (in the interior thigh) administered with serial 10-fold dilutions of *Y. pestis* (10^3 to 1 c.f.u., four mice for one dose) of 2-day agar cultures grown at 28 °C. Humane end points were strictly observed. Animals that succumbed to infection were sacrificed and examined bacteriologically. The remaining animals were observed for 21 days. The animals that survived were humanely killed by carbonic gas inhalation. The LD₅₀ and 95% confidence intervals were determined according to the method of Kärber (Finney, 1978). Mortality times were recorded and the mean times to death were calculated, taking into account all challenge doses for each strain.

For colonization/dissemination analysis, 10 mice were infected for each time point with the wild-type strain or its Δ *psaEFABC* variant by the s.c. route in the interior thigh (10^2 c.f.u.). At various times after infection (24, 36, 48 or 72 h), mice were euthanized by carbonic gas inhalation and superficial inguinal lymph nodes and spleens were removed and weighed. The bacterial load for each organ was determined by plating dilutions of the macerated tissues onto BHI agar plates; these were reported as c.f.u. (g tissue)⁻¹. Infections were repeated in at least two independent experiments.

RESULTS AND DISCUSSION

Construction and complementation of *Y. pestis* pH 6⁻ mutants

It is well-known that microbial pathogenesis is usually complex and multifactorial. Several virulence factors may act individually or in concert to produce infection and removal of any one of these components may or may not render the organism avirulent (Finlay & Falkow, 1997). To study the role of individual factors in the pathogenicity of *Y. pestis* and to reveal their possible involvement in the mechanisms that enable intracellular dissemination and reproduction of the plague pathogen in plasma and interstitial fluid, and within phagolysosomes, the use of genetically defined isogenic variants of the virulent strain is necessary.

Conflicting results were generated by testing the virulence of *Y. pestis* strains carrying mutations in different genes of the *psa* operon when using different routes of infection and involving parent strains differing in virulence and geographical origin (Cathelyn *et al.*, 2006; Lindler *et al.*, 1990;

Fig. 1. SDS-PAGE (a) and Western blot (b) analyses (Yang *et al.*, 1996) with anti-pH 6, showing the presence of pH 6 antigen in *Y. pestis* cell lysates (cultured at 37 °C, pH 5.8). Lanes: 1, purified F1 antigen (2 µg); 2, I-1996; 3, I-1996 Δ *psaA*; 4, 231; 5, 231 Δ *psaEFABC*; 6, purified pH 6 antigen (2 µg).

Panfertsev *et al.*, 1991). In the present study, two highly virulent biovar antiqua strains, 231 and I-1996, were used. The former was previously used in similar experiments (Panfertsev *et al.*, 1991) and the nucleotide sequence of its *psa* operon (GenBank accession number EF079883) was shown to be identical to those from the other completely sequenced *Y. pestis* strains (<http://www.eicbrc.org/portal/eric/yersiniapestis?id=enteropathogens&subid=yersiniapestis>).

We performed mutagenesis with suicide vectors pCVD442*ΔpsaA::kan* and pCVD442*ΔpsaEFABC::kan* to generate I-1996 Δ *psaA*, I-1996 Δ *psaEFABC* and 231 Δ *psaEFABC* knock-out mutants. Immunochemical tests, double diffusion analysis in agar gel, Western blot and ELISA with rabbit anti-pH 6 antigen sera indicated that they did not produce pH 6 antigen (Figs 1, 2 and 3).

The introduction of the plasmids pJG428 (*psaEFABC*) or pIG924Cm (*psaABC*) into *Y. pestis* mutants deficient in the complete *psaEFABC* operon resulted in complementation of the mutation. According to the data from immunochemical reactions, the strains complemented with

Fig. 2. Estimation of pH 6 antigen production in *Y. pestis* (cultured at 37 °C, pH 5.8) by dot-ELISA (Chandrika *et al.*, 1998), in strains 231 (line A), 231 Δ *psaEFABC*pIG924Cm (line B) and 231 Δ *psaEFABC*pJG428 (line C). Samples were diluted 1/1, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512 and 1/1024 (lanes 1–11, respectively).

Fig. 3. Estimation of pH 6 antigen production in *Y. pestis* by double diffusion analysis in agar gel (Öuchterlony, 1949). Samples were *Y. pestis* strains 231 (37 °C, pH 5.8), 231Δ*psaEFABC*pIG924Cm (37 °C, pH 5.8), 231Δ*psaEFABC*pIG924Cm (28 °C, pH 5.8), 231Δ*psaEFABC*pJG428 (37 °C, pH 5.8), purified pH 6 antigen, 231Δ*psaEFABC* (37 °C, pH 5.8), 231Δ*psaEFABC*pIG924Cm (37 °C, pH 7.2), 231Δ*psaEFABC*pJG428 (37 °C, pH 7.2), 231Δ*psaEFABC*pIG924Cm (28 °C, pH 7.2), 231Δ*psaEFABC*pJG428 (28 °C, pH 7.2), 231 (37 °C, pH 7.2), 231Δ*psaEFABC* (37 °C, pH 7.2) and anti-pH 6 (in wells 1–13, respectively).

pIG924Cm plasmid showed the lowest levels of pH 6 antigen production (Fig. 2). The wild-type strains produced approximately double the amount of antigens compared with the representatives of the strains complemented with pIG924Cm. The mutants carrying plasmid pJG428 produced nearly twice as much as the latter strains. In both *Y. pestis* Δ*psaEFABC* mutants, as well as in *E. coli* (data not shown), the plasmid pIG924Cm deficient in the regulatory genes *psaE* and *psaF*, was able to ensure temperature- (28–37 °C) and pH- (5.8–7.2) independent synthesis of pH 6 antigen (Fig. 3). In the absence of the regulator the functional promoter (–35 element, ATAGCA; –10 element, GACTGT), located before the three coding sequences (*psaABC*) within the remaining *SauI*–*Clai*-fragment of the *psa* operon, becomes functional and ensures constitutive gene expression.

Animal passage of *Y. pestis* pH 6[–] mutants and complemented strains

After the first round of passage of *Y. pestis* 231Δ*psaEFABC*pJG428, the Ap^R and pH 6⁺ cultures were isolated from 50 % (two of four), and after the second round from 100 %, of the animals that succumbed to infection. In contrast, we failed to isolate the Ap^R *Y. pestis* clones from the animals infected with I-1996Δ*psaEFABC*pJG428. These results are in agreement with the suggestion that transfer of genetic material might be more frequent in atypical forms of *Y. pestis* with reduced virulence (Domaradskii, 1987).

After the first passage of *Y. pestis* 231Δ*psaEFABC*pIG924Cm and I-1996Δ*psaEFABC*pIG924Cm, 100 % of the isolated cultures were Cm^R. The second round of passage in naïve mice did not eliminate the plasmid either; 100 % of the isolated cultures of both strains were Cm^R and pH 6⁺.

Interestingly, after the second round of animal passage of complemented strains that was performed without antibiotic treatment, elimination of recombinant plasmids carrying the *psa* operon was not detected. Previously, it was shown that plasmids containing the ColE1 replicon were

not able to maintain stability in *Y. pestis* recipient cells without selective pressure (Drozdoz *et al.*, 1995). Similarly, 94–96 % of the complemented mutants isolated from animals in antibiotic-free BHI broth at 37 °C lost pJG428 or pIG924Cm plasmids (data not shown). A similar stabilization of plasmid pFS1 coding for another *Y. pestis* pilus adhesin, capsular antigen F1, which was also constructed using pHC79, was observed in *Salmonella enterica* bv. Typhimurium strain SL3261pFS1 after passage in mice (Gremyakova, 2004). Moreover, in our experiments, analysis of bacterial colonization/dissemination in infected animals indicated that wild-type bacteria replicated more rapidly in lymph nodes compared with their pH 6[–] derivative, but this was not the case in the spleen. Hence, selection in the mammalian host for the pH 6⁺ or F1⁺ clones offers a selective advantage for bacterial cells producing pilus adhesins and was the reason why virulence of all recombinant strains obtained in this study was estimated without antibiotic treatment of experimentally infected animals. This decision was supported by isolation of clones carrying pJG428 or pIG924Cm plasmids, coding for pH 6 antigen, from the organs of all the mice that succumbed to infection.

Virulence of *Y. pestis* strains differing in ability to produce pH 6 antigen

All pH 6-immunized animals possessed high antibody titres to pH 6 antigen: 1/5120–1/10 240 (mean antibody titres=1/8960). Comparative study of the virulence of *Y. pestis* mutant strains using subcutaneously challenged mice did not reveal differences in their LD₅₀ (Table 2). The average survival time of mice that succumbed to infection with strains 231 and I-1996 or their isogenic derivatives did not differ from each other. Analysis of *Y. pestis* cultures isolated from animals that succumbed to infection indicated that pJG428 or pIG924Cm were stably maintained *in vivo*: 100 % of the isolated clones were Ap^R pH 6⁺ or Cm^R pH 6⁺, respectively.

At 48 h after s.c. inoculation, the weight of lymph nodes from mice infected with wild-type strain 231 was

Table 2. LD₅₀ and mean survival time in mice infected subcutaneously with *Y. pestis* strains

ND, Not determined.

<i>Y. pestis</i> strain	pH 6 antigen	Naïve mice		Immune mice	
		LD ₅₀ (c.f.u.)	Mean time to death (days)*	LD ₅₀ (c.f.u.)	Mean time to death (days)*
231	+	1 (1–2)	5.7 ± 1.48	2 (1–10)	5.9 ± 1.56
231Δ <i>psaEFABC</i>	–	2 (1–5)	5.5 ± 1.10	1 (1–2)	5.9 ± 1.56
231Δ <i>psaEFABC</i> pIG428	+	3 (1–10)	4.9 ± 1.15	ND	ND
231Δ <i>psaEFABC</i> pIG924Cm	+	3 (1–10)	5.5 ± 1.80	2 (1–7)	5.8 ± 1.37
I-1996	+	1 (1–2)	6.2 ± 1.56	4 (1–15)	6.4 ± 1.19
I-1996Δ <i>psaA</i>	–	4 (1–16)	5.7 ± 1.10	ND	ND
I-1996Δ <i>psaEFABC</i>	–	3 (1–13)	6.1 ± 1.36	1 (1–2)	6.1 ± 1.36
I-1996Δ <i>psaEFABC</i> pIG924Cm	+	1 (1–5)	7.1 ± 2.21	1 (1–5)	7.2 ± 2.12

*Values are given ± 95 % confidence intervals.

significantly higher ($P < 0.05$) than in animals challenged with its Δ*psaEFABC* variant (Table 3). In wild-type-infected animals, the first bacteria could be isolated from lymph nodes at 48 h, and at 72 h the bacterial load reached its maximum within the time period studied ($\log_{10}$ c.f.u. g⁻¹, 6.2 ± 1.2). In the Δ*psaEFABC* mutant-infected mice, the first bacteria could be isolated from lymph nodes only at 72 h ($\log_{10}$ c.f.u. g⁻¹, 2.5 ± 2.2). There were no significant differences ($P < 0.05$) in the weight of spleens and the rate of bacterial colonization/dissemination in animals inoculated with the wild-type or its Δ*psaEFABC* mutant, though levels of *Y. pestis* dissemination were slightly lower in Δ*psaEFABC* mutant-infected animals compared with wild-type-infected animals ($\log_{10}$ c.f.u. g⁻¹, 4.7 and 5.6, respectively). Slower dissemination of the Δ*psaEFABC* cells at the early stages of infection might have caused rapid multiplication at the inoculation site, with the consequence of a large number of bacteria compensating for the initial dissemination delay. It is well-known that the spleen is an immunological tissue that increases in

size during infection (Bowdler, 2001). However, the weight of the spleens from animals infected with both pH 6⁺ and pH 6⁻ bacteria initially decreased (up to 48 h) and then increased at 72 h, coinciding with the appearance of bacteria in this organ (Table 3). Most likely, the early spleen diminution might be a result of redistribution of blood from the splenic blood depot (Kovalev, 1978) to the initial site of bacterial replication.

Previous studies have also shown that the loss of capsular antigen F1 (Du *et al.*, 2002), representative of the family of pilus adhesins (Karlyshev *et al.*, 1994), did not affect virulence of *Y. pestis* (Drozdov *et al.*, 1995; Friedlander *et al.*, 1995). Site-directed mutations affecting F1 production did not decrease *Y. pestis* virulence in mice (Drozdov *et al.*, 1995; Friedlander *et al.*, 1995), guinea pigs (Drozdov *et al.*, 1995) or monkeys (Friedlander *et al.*, 1995). Probably, in the case of the loss of any of these antigens, the mutations were functionally complemented by increase in production of one or several of the eight other pilus

Table 3. Mean weight of organs from subcutaneously infected mice and bacterial loads in these organs at different times after infection

Values shown are means ± SD. Values in bold type within the same column are significantly different ($P < 0.05$) from each other, as calculated by Student's *t* test.

<i>Y. pestis</i> strain	Time after infection (h)	Lymph nodes		Spleens	
		Mean weight (mg)	$\log_{10}$ c.f.u. g ⁻¹	Mean weight (mg)	$\log_{10}$ c.f.u. g ⁻¹
231	24	52.4 ± 15.9	0	150.5 ± 26.3	0
	36	51.9 ± 11.3	0	125.7 ± 15.9	0
	48	74.6 ± 13.3	2.4 ± 1.3	106.4 ± 19.9	0
	72	72.3 ± 13.8	6.2 ± 1.2	130.7 ± 27.6	5.6 ± 1.8
231Δ <i>psaEFABC</i>	24	38.3 ± 7.8	0	154.3 ± 75.3	0
	36	40.8 ± 12.4	0	134.0 ± 21.6	0
	48	42.9 ± 10.4	0	109.7 ± 7.7	0
	72	66.6 ± 15.6	2.5 ± 2.2	116.7 ± 22.2	4.7 ± 1.4

adhesins which were revealed in the *Y. pestis* genome (Felek *et al.*, 2007; Parkhill *et al.*, 2001). Our proposal is supported by the report that the *psaABC* genes of *Y. pestis* were able to functionally complement mutation in *Pseudomonas aeruginosa* pilus adhesin and restore cytotoxicity of the pathogen by ensuring contact with the target eukaryotic cell necessary for type III secretion of the effector protein, exoenzyme S (Sundin *et al.*, 2002).

The data presented in this paper on the role of pH 6 antigen in 'full' virulence of *Y. pestis* are seemingly in conflict with the results of earlier investigations (Cathelyn *et al.*, 2006; Lindler *et al.*, 1990; Panfertsev *et al.*, 1991). Thus, studies of the attenuated Pgm⁻ strain KIM5 (Lindler *et al.*, 1990) allow us to speculate that the 200-fold decrease in virulence of pH 6⁻ derivative in intravenous (i.v.) mouse infection might have been caused by the low virulence of the parent strain used. In addition, this strain was only able to cause lethal systemic infection following i.v. challenge. In our opinion, accumulation of mutations, which potentially reduce the ability of the wild-type strain to multiply in the host organism, may be compensated to some extent by the presence of a large number of pathogenicity factors in *Y. pestis*. Stepwise elimination of these factors can no longer compensate their loss at the expense of overproduction of the residual virulence factors and become apparent as a noticeable decrease of virulence.

It is reported that an approximately 80-fold drop in LD₅₀ and reduced dissemination/colonization of spleens and lungs in mice after s.c. infection with Δ rovA mutant of *Y. pestis* wild-type strain CO92 correlated with the decrease in expression of the *psa* operon (Cathelyn *et al.*, 2006). The Δ psaA mutation in CO92 resulted in a similar dissemination defect after s.c. challenge. These data are not shown in this paper.

We can propose several explanations for the case of the striking differences in virulence of pH 6⁻ mutants generated from strain 231. We can not rule out that Panfertsev *et al.* (1991) used a non-animal-passaged laboratory subcultivated clone of the initial strain 231 in their experiments. Due to repeated reseeds on nutrient media, it is possible that the bacterial population may have acquired unidentified mutations (modifications) which potentially reduced the ability of the strain to survive in the host organism. The loss of one more pathogenicity factor, pH 6 antigen, caused the complete loss of subcutaneous virulence in mice.

Comparing the results obtained using *Y. pestis* strains of different origin, one should consider the significance of individual pathogenicity factors in the virulence of various *Y. pestis* intraspecies groups (biovars, subspecies, ecotypes, etc.) for various animal species (Anisimov, 2002a). These strain differences result from microevolutionary adaptation of geographically separated *Y. pestis* in specific rodent species and they are characterized by significant individual metabolic traits. Taking this into account, it may be necessary to include in our experiments all of the previously studied parent strains, which exhibit different

levels of attenuation after introduction of mutations into the *psa* operon. However, the current laws, regulations and restrictions in force on international exchange of highly pathogenic microbes made it practically impossible to use all the desired strains in this study.

Previous experiments on active immunization of laboratory animals with preparations of pH 6 antigen provide evidence of the absence of its protective properties in the presence of a pronounced ability to induce antibody formation (Gremyakova, 2004; Titball & Williamson, 2001; Vodop'ianov *et al.*, 1995) and are in accordance with our current results. We speculate that the absence of protective activity is associated with peculiarities in the pH regulation of production of this antigen. Synthesis of pH 6 antigen by *Y. pestis* cells inside phagolysosomes of the macrophages or in the centre of necrotically degenerated tissues of abscesses (Ben-Efraim *et al.*, 1961) should make pH 6⁺ bacteria unable to interact with antibodies and immunocompetent cells. Accordingly, the induction of an immune response is like a 'blank shot', directing the immune system toward an unachievable goal and exhausting its resources. If this is true, pH-independent production of pH 6 antigen should make *Y. pestis* Δ psaEFABCpIG924Cm variants susceptible to killing by immunity induced with pH 6 antigen. However, high antibody titres to pH 6 antigen did not correlate with protection even in the group of mice challenged with the strains producing this antigen permanently. These results may explain the absence of reports on isolation of pH 6⁻ strains from natural plague foci and experimentally infected animals. F1⁻ strains are not rare, due to the high protective activity of F1 antigen which leads to selection of non-capsulated *Y. pestis* derivatives in immunized or surviving plague-infected animals (Anisimov, 2002b; Anisimov *et al.*, 2004; Friedlander *et al.*, 1995; Perry & Fetherston, 1997). Thus, antiphagocytic activity, together with the presence of antigen on the bacterial cell surface, cannot guarantee the same protective potency of similar microbial antigens.

Concluding remarks

The main outcome of our investigation is the finding that the loss of the ability to produce pH 6 antigen did not influence virulence of Δ psaA or Δ psaEFABC mutants of *Y. pestis* in the case of s.c. challenge of naïve and immune mice. This argues against the usefulness of using pH 6 antigen as a molecular target for plague prophylaxis and therapy. However, pH 6 antigen, or most likely genes from the *psa* operon, may be useful targets for laboratory diagnosis of infections caused by *Y. pestis* and/or *Y. pseudotuberculosis*.

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