Evaluation of O-antigen inactivation on Pla activity and virulence of *Yersinia pseudotuberculosis* harbouring the pPla plasmid

Flavie Pouillot,¹ Anne Derbise,¹ Maini Kukkonen,² Jeannine Foulon,¹ Timo K. Korhonen² and Elisabeth Carniel¹

Correspondence

Elisabeth Carniel
carniel2@pasteur.fr

¹*Yersinia* Research Unit, Institut Pasteur, 28 rue du Dr Roux, 75724 Paris Cedex 15, France

²General Microbiology, Faculty of Biosciences, FIN-00014 University of Helsinki, Finland

Yersinia pestis is a species that emerged recently from *Yersinia pseudotuberculosis* and gained an exceptional pathogenicity potential. Among the major genetic differences between the plague bacillus and its ancestor is the acquisition of the pPla plasmid, which has been associated with the increased virulence of *Y. pestis*. In a previous study, introduction of pPla into *Y. pseudotuberculosis* did not lead to any modification of the virulence of the host bacterium. However, it was subsequently demonstrated that the presence of smooth lipopolysaccharide (LPS) inhibits the activity of Pla. In this study, pPla was introduced into a *Y. pseudotuberculosis* strain expressing smooth LPS, and into a variant in which a mutation that abrogates the formation of O-antigen (O-Ag) repeats (as in natural isolates of *Y. pestis*) was generated. It was found that in both strains, Pla was synthesized, exported to the bacterial membrane and processed as in *Y. pestis*. However, the ability of Pla to activate plasminogen was weak and observed only at 37 °C in the smooth strain, while this activity was similar to that of *Y. pestis* and expressed at both 28 and 37 °C in the O-Ag mutant strain. Similarly, Pla-mediated inactivation of the antiprotease α_2 -antiplasmin was not detected in the smooth *Y. pseudotuberculosis* strain grown at 28 °C, but was expressed at both temperatures in the O-Ag mutant strain. Despite the more efficient activity of Pla, the *Y. pseudotuberculosis* O-Ag mutant strain exhibited a lower pathogenicity upon subcutaneous infection of mice. The results thus indicate that, although abrogation of O side chain synthesis in a *Y. pseudotuberculosis* strain harbouring pPla potentiates the two proteolytic activities of Pla, this is not sufficient to confer to *Y. pseudotuberculosis* a higher pathogenicity potential. These results also suggest that acquisition of pPla may not have been sufficient to confer an immediate higher pathogenic potential to the ancestor *Y. pestis* strain.

Received 16 June 2005
Revised 10 August 2005
Accepted 26 August 2005

INTRODUCTION

Yersinia pestis has caused at least three waves of pandemic plague of devastating magnitude. The bacterium still persists endemically in many countries of Africa, Asia and America and is responsible for local outbreaks (World Health Organization, 2003). This Gram-negative bacterium is transmitted from rodent to rodent, and from rodent to human by fleabites. After injection into the dermis of a new host, *Y. pestis* rapidly disseminates to the draining lymph node, the spleen and the liver, and produces an often fatal systemic infection.

Y. pestis is a highly uniform species, which emerged about 1500 to 20 000 years ago from *Yersinia pseudotuberculosis*

(Achtman *et al.*, 1999), probably from a serotype O:1b strain (Skurnik *et al.*, 2000). Despite their very close genetic relationship, the two species have distinct epidemiological and clinical features, and a drastically different pathogenic potential. The molecular bases for their difference in virulence are still not understood. Comparison of the genome sequences of representatives of the two species revealed an extensive genetic loss that has probably been more important than acquisition of new genes in the evolution of *Y. pestis* (Chain *et al.*, 2004). Nonetheless, 32 *Y. pestis* chromosomal genes and two *Y. pestis*-specific plasmids have been acquired by *Y. pestis* since its divergence from *Y. pseudotuberculosis*. The two *Y. pestis*-specific plasmids are a 101 kb plasmid called pFra (or pMT1) and a 9.6 kb plasmid called pPla (or pPst or pPCP1).

pFra encodes a phospholipase D (Hinnebusch *et al.*, 2000; Rudolph *et al.*, 1999), which promotes *Y. pestis* survival in and colonization of the flea midgut (Hinnebusch *et al.*,

Abbreviations: α_2 AP, α_2 -antiplasmin; ECM, extracellular matrix; LPS, lipopolysaccharide; O-Ag, O-antigen; sc, subcutaneous; Tmp, trime-thoprim.

2002). The fact that a pFra-cured *Y. pestis* is still fully virulent for mice (Friedlander *et al.*, 1995) and African green monkeys (Davis *et al.*, 1996), indicates that the main property of this plasmid is not to enhance *Y. pestis* virulence but to support its flea-borne transmission.

The pPla plasmid encodes at least four proteins (Sodeinde & Goguen, 1988): the bacteriocin pesticin (Pst), its immunity protein (Pim), an IS100 transposase and a plasminogen activator (Pla). Activation of plasminogen by Pla damages the extracellular matrix (ECM) of cultured human epithelial cells (Lähteenmäki *et al.*, 1998). Plasminogen is a circulating precursor of the serine protease plasmin, whose main physiological function is to degrade fibrin clots and ECMs, thus facilitating cell migration (Plow *et al.*, 1999; Saksela, 1985). Pla not only increases plasmin activity by cleaving plasminogen, but also inactivates the antiprotease α_2 -antiplasmin (α_2 AP), which is the main inhibitor of plasmin (Kukkonen *et al.*, 2001). Pla has been shown to play a role in *Y. pestis* virulence. Some *pla* mutants of *Y. pestis* were severely attenuated (one millionfold) after subcutaneous (sc) infection of mice (Sodeinde *et al.*, 1992). It has been proposed that Pla facilitates *Y. pestis* dissemination from its site of inoculation by cleaving fibrin deposits that trap the organisms, and by reducing the chemoattraction of inflammatory cells (Sodeinde *et al.*, 1992). However, in another study, no difference in the bacterial proliferation and local inflammatory response induced by wild-type *Y. pestis* or the *pla* mutant strain was observed at the site of injection, but a lower number of *pla* mutants at more distant sites, such as the draining lymph node and spleen, was noted (Welkos *et al.*, 1997). Furthermore, a few *Y. pestis* isolates lacking pPla were found to be fully virulent (Kutyrev *et al.*, 1989; Samoilova *et al.*, 1996; Welkos *et al.*, 1997), indicating that the role of pPla in *Y. pestis* pathogenesis remains to be clarified.

To determine whether acquisition of pPla may have been sufficient to confer an increased pathogenicity potential to the *Y. pestis* ancestor, Kutyrev *et al.* (1999) introduced this plasmid into *Y. pseudotuberculosis* to evaluate the pathogenicity of the recombinant strains for mice infected subcutaneously. Although Pla was produced and expressed on the cell surface, no significant modification of the LD₅₀ of the pPla harbouring *Y. pseudotuberculosis* recombinant strains, as compared to the wild-type strain, was observed, suggesting that the presence of this plasmid is not sufficient to confer additional pathogenicity potential to the host strain. However, it was subsequently demonstrated that O-antigen (O-Ag) repeats in the lipopolysaccharide (LPS) of *Y. pseudotuberculosis* sterically inhibit plasminogen activation by Pla (Kukkonen *et al.*, 2004). The lack of Pla activity due to the presence of O-Ag repeats on the surface of *Y. pseudotuberculosis* could thus have accounted for the previously observed absence of increased pathogenicity of the pPla harbouring *Y. pseudotuberculosis* strains.

In this study, a mutation that abrogates the formation of O-Ag repeats (as in natural isolates of *Y. pestis*) was introduced

into the chromosome of a pPla-harbouring *Y. pseudotuberculosis* strain. In this background, the ability of Pla to activate plasminogen and to degrade α_2 AP was investigated, and the impact of pPla on the virulence of the recombinant strain for mice was evaluated.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were grown in Luria–Bertani (LB), brain–heart infusion or Müller–Hinton (MH) broth, and on LB, LB-Sac (without NaCl and supplemented with 10% sucrose) or MH agar. *Yersinia* strains were grown at 28 or 37 °C, and *Escherichia coli* strains at 37 °C. Chloramphenicol (25 µg ml⁻¹), kanamycin (30 µg ml⁻¹) or trimethoprim (Tnp; 100 µg ml⁻¹) were added to the media as necessary. Selection for Tmp resistance was done in MH broth. All experiments involving virulent *Y. pestis* CO92 were performed in a BL3 laboratory.

DNA and gene manipulation. Genomic and plasmid DNA were extracted with the Isoquick nucleic acid extraction kit, and the Qiagen Plasmid Maxi Kit, respectively. Mutagenesis of chromosomal target genes and insertion of antibiotic-resistance genes onto the plasmid were performed as described previously (Derbise *et al.*, 2003). Recombinant plasmid DNA and PCR fragments were introduced into *E. coli* by transformation or into *Yersinia* by electroporation, as described by Conchas & Carniel (1990).

PCR. The primers used in this study are listed in Table 2. PCRs were performed with 1 unit of *Taq* polymerase (Roche) or 1 unit of a 3:1 mixture of *Taq* and *Pfu* (Stratagene) polymerases in the supplier's buffer. PCR amplification reaction mixtures contained 10 µM each primer and 1 mM dNTPs. The PCR program involved one step at 95 °C for 5 min, followed by 30 cycles of amplification of three steps at (i) 95 °C for 30 s, (ii) 55 °C for 30 s and (iii) 72 °C for 1–3 min, depending on the fragment length. PCR products were maintained at 72 °C for 5 min, electrophoresed in 1% agarose gels, and stained with ethidium bromide. Amplification of the *kan* cassette with long flanking homologous regions of the *Yersinia* target DNA was done with the PCR program described by Derbise *et al.* (2003).

Generation of mutant strains. Labelling of pPla with a *dfr* cassette encoding resistance to Tmp was carried out following the (short flanking homologous regions) SFH-PCR procedure (Derbise *et al.*, 2003). Briefly, the *dfr* locus was amplified by PCR with the primer pair 258A/258B, using pGP704N-*dfr* (Lesic & Carniel, 2005) as a template. The amplification product consisted of the entire *dfr* gene and promoter region, flanked by 55 bp homologous to an intergenic region on pPla (I-Region). This PCR product was introduced into *Y. pestis* CO92 (pKOBEG-*sacB*) by electroporation. Colonies in which allelic exchange between the linear PCR product and the plasmid target sequence had occurred were selected on agar plates containing Tmp. Correct insertion of the *dfr* cassette was checked by PCR with primers 260B/260C, which are located on each side of the inserted fragment (Fig. 1a).

Introduction of pPla into *E. coli* LE392 and *Y. pseudotuberculosis* IP32953 was done by electroporation and mutants were selected on Tmp plates. The integrity of the plasmid was checked by digestion with *Bam*HI, yielding the expected 9.9 kb linear plasmid fragment.

To obtain a *Y. pseudotuberculosis* strain that does not synthesize O side chains, primer pairs OKF/OKf1 and OKR/OKr2 were used to amplify the region encompassing the *gmd* and *fcl* genes, and primer pair 136A/136B to amplify the *kan* cassette (Table 2). Following the (long flanking

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic	Source
<i>Y. pseudotuberculosis</i>		
IP32953	Serotype I (France)	Institut Pasteur
IP32953(pKOBEG- <i>sacB</i>)	pKOBEG- <i>sacB</i> introduced into IP32953 by electroporation	This study
IP32953 O-Ag ⁻	Mutations in the <i>gmd</i> and <i>fcl</i> genes that abrogate the formation of O-Ag repeats	This study
IP32953(pPla)	pPla-Tmp introduced into IP32953 by electroporation	This study
IP32953(pPla) O-Ag ⁻	pPla-Tmp introduced into IP32953 O-Ag ⁻ by electroporation	This study
<i>Y. pestis</i>		
CO92	Wild-type, biotype Orientalis	USA
CO92(pKOBEG- <i>sacB</i>)	pKOBEG- <i>sacB</i> introduced into CO92 by electroporation	This study
CO92(pPla)	pPla-Tmp introduced into CO92(pKOBEG- <i>sacB</i>) by electroporation	This study
<i>E. coli</i>		
LE392	<i>supE44 supF58 hsdR514 galK2 galT22 metB1 trpR55 lacY1</i>	BioLabs
LE392(pPla)	pPla-Tmp introduced into LE392 by electroporation	This study
Plasmids		
pKOBEG- <i>sacB</i>	<i>repA cat araC pBAD exo bet gam sacB</i> , Cm ^R	Derbise <i>et al.</i> (2003)
pUC4K	<i>bla oriR6K oriT cos rpsL kan</i> , Amp ^R , Kan ^R	Amersham
pGP704N- <i>dfr</i>	<i>oriR6K mobRP4 bla dfr</i> , Amp ^R , Tmp ^R , suicide vector	Lesic & Carniel (2005)
pPla-Tmp	pPla plasmid of <i>Y. pestis</i> with a <i>dfr</i> cassette inserted in the I-Region; Tmp ^R	This study

Table 2. Primers used for PCR amplification

Lower-case letters correspond to sequences located on the *kan* (primer pair OKf1/OKr2) or *dfr* (primer pair 258A/258B) antibiotic-resistance genes.

Primer	Target gene	Nucleotide sequence
18	<i>irp2</i>	5'-GCCGGAAGCCTGGCCTTTA-3'
19		5'-CTATGCTGACAGTTGAAGGC-3'
37A	<i>cafI</i>	5'-CAGTTCCGTTATCGCCATTGC-3'
37B		5'-TATTGGTTAGATACGGTTACGGT-3'
159A	<i>pla</i>	5'-ATCTTACTTTCGTTGAGAAG-3'
159B		5'-CTTGGATGTTGAGCTTCCTA-3'
160A	<i>yopM</i>	5'-ATAACTCATCGGGGGCAAAAT-3'
160B		5'-GCGTTATTATCCGAATTTAGC-3'
258A	<i>I-Region-dfr</i>	5'-CACAAATCTCTAATCCCGACAAGGTCGTAGGTCGTTA-TAGGAAAATTCTTtgagctgttgacaattaac-3'
258B		5'-GCGTTTTTATATTCGTAATGTTGTCAGTCGTTTCATGG-CCTGCTGTTCTGattgagatccaacaccaac-3'
260B	Flanking the <i>I-Region</i>	5'-TCACACTCTTCCGCTAATCC-3'
260C		5'-CAAGAATGCCGAGATACGCAGTCA-3'
136A	<i>kan</i>	5'-GGGATAAGTGTATCAAGAAACC-3'
136B		5'-GCTCCTCCAGCCATAATTACAGG-3'
166	<i>kan</i> (outward)	5'-TCCTATGGAAGTGCCTCGGT-3'
167		5'-ATCGCGAGCCCATTTATACC-3'
OKup	Flanking <i>gmd</i> and <i>fcl</i>	5'-GTGAAAGTCTAAACTTATTGGC-3'
OKdown		5'-GCATCGTCAAATCTGACGTTAGCGC-3'
OKF	<i>gmd-kan</i>	5'-GGGATAAGTGTATCAAGAAACCT-3'
OKf1		5'-gattttgagacacacgtggctttGCATACATTTTGGCAACAGC-3'
OKR	<i>fcl-kan</i>	5'-GCACCTCCAGCCATAATTACAGG-3'
OKr2		5'-cgatgagtttttctaatacagaTAAACCAATGCGTGAGTTCCTGC-3'

Fig. 1. Genetic maps of pPla, and the LPS chromosomal locus with the sites of allelic exchange between the target gene and an antibiotic-resistance cassette. (a) pPla with the insertion of a Tmp-resistance cassette in the I-Region. (b) The O-Ag gene cluster with the replacement of the *gmd* and *fcl* loci by a *kan*-resistance cassette. Arrows indicate the location and direction of the primers used to check proper allelic exchanges.

homologous regions) LFH-PCR procedure (Derbise *et al.*, 2003), a linear PCR fragment composed of the *kan* gene with its promoter region, and flanked by ≈ 550 bp DNA homologous to the regions located on each side of *gmd* and *fcl*, was generated. This fragment was introduced by electroporation into strain IP32953(pKOBEG-*sacB*) and correct allelic exchange between the PCR fragment and the chromosomal gene was checked by PCR with primer pairs OKup/167, OKdown/166 and OKup/OKdown (Table 2, Fig. 1b). *Y. pseudotuberculosis* IP32953 clones with the appropriate O-Ag mutation and cured of pKOBEG-*sacB* were selected on LB-Sac agar plates.

The IP32953(pPla) O-Ag⁻ variant was obtained by introducing pPla-Tmp into IP32953 O-Ag⁻ by electroporation. Presence of a plasmid with the appropriate size was checked by digestion of the plasmid extract with *Bam*HI.

Protein analysis. To determine whether Pla is present in bacterial cell-envelope preparations, bacteria grown overnight were centrifuged, and cell suspensions were adjusted to an OD₆₀₀ 1.2 in PBS. Bacteria were sonicated four times for 30 s over crushed ice. Unbroken cells were removed by low speed centrifugation (1800 g), and the cell envelopes were pelleted by centrifugation at 15 000 g for 10 min at 4 °C. Total membrane proteins were separated by SDS-PAGE in 12.5 % gels, the proteins were transferred onto PVDF membranes (Hybond), and the Pla polypeptides were detected using 1:500 dilutions of primary anti-His₆-Pla antisera (Kukkonen *et al.*, 2001) and 1:10 000 dilutions of secondary anti-rabbit-IgG alkaline-phosphatase conjugate. Bound antibodies were detected with the phosphatase substrate (ECL Plus Kit, Amersham).

Extraction and analysis of LPS. Bacteria grown overnight at 37 or 25 °C on LB plates were collected and resuspended in 2 ml PBS. The bacterial suspension was adjusted to OD₄₂₀ 0.4 and subjected to

proteolysis for 2 h at 60 °C by the addition of 40 µg proteinase K ml⁻¹. An aliquot (1.5 ml) of this suspension was centrifuged for 3 min at 1 200 g, and the pellet was resuspended in 50 µl lysis buffer [2 % SDS, 4 % β-mercaptoethanol, 10 % (v/v) glycerol, 1 M Tris-HCl pH 6.8, bromophenol blue] and heated at 100 °C for 10 min. Ten microlitres of this extract was subjected to SDS-PAGE in a 15 % polyacrylamide gel and transferred onto PVDF membranes. The LPS was detected using 1:1000 dilution of a primary rabbit anti-LPS antiserum raised against *Y. pseudotuberculosis* V2812/79 (kindly provided by Dr M. Skurnik, University of Helsinki, Helsinki, Finland), and 1:10 000 dilution of secondary anti-rabbit IgG alkaline-phosphatase conjugate. Bound antibodies were detected with the phosphatase substrate.

Plasminogen activation and α₂AP degradation. Kinetic measurement of plasminogen activation was performed as described by Kukkonen *et al.* (2001), by incubating 2×10^8 bacteria, 4 µg human Glu-plasminogen (American Diagnostica) and the chromogenic plasmin substrate S-2251 (Val-Leu-Lys-p-nitroaniline dihydrochloride; Chromogenix) in a total volume of 200 µl at 37 °C. Breakdown of the chromogenic substrate was measured in a microtitre-plate reader at A₄₀₅. The A₄₀₅ values were determined at time intervals of 15 min. The level of α₂AP inactivation was measured as described by Lähteenmäki *et al.* (2005). Briefly, 5 µg α₂AP ml⁻¹ was incubated overnight at 37 °C with 10^9 bacteria in 105 µl PBS. Gentamicin (1 µg ml⁻¹) was added to prevent bacterial growth. After the addition of 100 µl PBS, bacteria were removed by centrifugation and 168 µl supernatant was transferred into a well of a microtitre plate. Human plasmin (2.5 µg ml⁻¹; Sigma) and the plasmin substrate S-2251 (0.45 mM; Chromogenix) were added, and plasmin activity was measured at A₄₀₅ as the breakdown of the substrate after 90 min incubation at 37 °C. The control for α₂AP activity was produced by

incubating α_2 AP with PBS instead of bacteria, and plasmin activity was checked in the absence of bacteria and α_2 AP. All measurements were carried out in duplicate and the experiments were repeated twice.

Animal infections. Prior to infection, the presence in each *Yersinia* strain of known unstable genetic elements, such as the plasmid(s) and the high-pathogenicity island, was systematically verified by PCR with primer pairs located on these various elements: 18/19 (*irp2*), 37A/37B (*caf1*), 159A/159B (*pla*) and 160A/160B (*yopM*) (Table 2). Groups of five 5-week-old OF1 female mice (Iffa Credo) were inoculated subcutaneously with 0.1 ml bacterial suspension. Lethality was recorded daily for 3 weeks. The LD₅₀ was determined according to the method of Reed & Muench (1935). To determine whether pPla-Tmp is stably maintained in *Y. pseudotuberculosis* during growth *in vivo*, three mice were infected subcutaneously with 10⁸ c.f.u. of the IP32953(pPla) recombinant strain. Moribund animals were euthanized, their spleens were removed aseptically, crushed in saline and streaked onto LB plates. Individual colonies (100) were spotted onto MH and MH-Tmp agar plates. The percentage of Tmp^R colonies were recorded. All experiments were performed in a biosafety level 3 animal facility.

RESULTS

Construction of *Y. pseudotuberculosis* recombinant strains carrying pPla-Tmp and/or a mutation in O-Ag biosynthesis genes

In order to introduce the pPla plasmid into *Y. pseudotuberculosis* IP32953, the *Y. pestis* CO92 pPla was first labelled with a *dfr* cassette inserted at position 2071–2091 on the plasmid sequence (Parkhill *et al.*, 2001). This insertion site, designated I-Region, was chosen because it is located between the IS100 coding sequence and the replication locus (Fig. 1a), in an apparently non-coding region. To ensure that this insertion did not affect *Y. pestis* virulence, *Y. pestis* CO92 strains carrying either the natural pPla or the labelled pPla-Tmp were injected subcutaneously into mice and their virulence was compared. The LD₅₀ of the *Y. pestis* strains harbouring the wild-type pPla (29 c.f.u.) and the labelled pPla-Tmp (32 c.f.u.) were similar, indicating that the presence of the antibiotic-resistance cassette did not affect pPla-virulence-associated properties. In addition to pPla, *Y. pestis* harbours two other resident plasmids: pFra and pYV. In order to purify the pPla-Tmp molecules present in *Y. pestis* CO92, the plasmid content of this strain was extracted and introduced into *E. coli* LE392 by electroporation. Plasmid extracts of Tmp^R *E. coli* were checked and subsequently used to introduce pPla-Tmp into *Y. pseudotuberculosis* IP32953 by electroporation, yielding IP32953(pPla).

Since the presence of O-Ag repeats was found to sterically inhibit plasminogen activation by Pla (Kukkonen *et al.*, 2004), a *Y. pseudotuberculosis* derivative with a mutation in the O-Ag biosynthesis pathway was constructed. For this purpose, most of the *gmd* and *fcl* genes which encode a GDP-mannose-4,6-dehydratase and a GDP-fucose synthetase, respectively, were replaced by a *kan* cassette (Fig. 1b). These two genes were chosen because they are naturally inactivated

in the O-Ag gene cluster of *Y. pestis* (Skurnik *et al.*, 2000), and they act at an early step of O-side-chain assembly (in the biosynthesis of the repeat unit sugar precursors). Absence of O-Ag synthesis in the resulting IP32953 O-Ag[−] mutant strain was evidenced by immunoblotting with an anti-LPS antibody. While the wild-type strain synthesized O side chains at 25 °C, the O-Ag mutant strain was unable to do so (Fig. 2). pPla-Tmp was subsequently introduced into IP32953 O-Ag[−], yielding IP32953(pPla) O-Ag[−].

Presence of Pla in *Y. pseudotuberculosis* membranes

Immunoblotting of bacterial membrane proteins with anti-His₆-Pla antibodies revealed the presence of Pla in the cell envelopes of both IP32953(pPla) and IP32953(pPla) O-Ag[−], in amounts similar to those observed in *Y. pestis*, and its absence in strains that do not carry pPla (Fig. 3). The presence of Pla in membranes was detected in bacteria grown at either 28 °C (data not shown) or 37 °C (Fig. 3). Pla is known to undergo C-terminal autoprocessing in *Y. pestis*, leading to two major forms designated α -Pla and β -Pla (Kukkonen *et al.*, 2001). Similar autoprocessed forms of Pla were detected in the recombinant *Y. pseudotuberculosis* strains harbouring pPla (Fig. 3). Therefore, as observed by Kuttyrev *et al.* (1999), when pPla is introduced into *Y. pseudotuberculosis*, Pla is synthesized, exported to the bacterial membrane and processed as in *Y. pestis*.

Fig. 2. Western blot of the LPS extract of the *Y. pseudotuberculosis* wild-type strain IP32953 and the O-Ag mutant strain grown at either 25 or 37 °C, detected with anti-LPS antibodies.

Fig. 3. Western blot of cell envelope proteins from *Yersinia* strains grown at 37 °C, detected with anti-His₆-Pla antisera. α -Pla is the mature form of the protein, β -Pla is autoprocessed at a single site at its C-terminus. Numbers on the left indicate the size of the molecular mass markers (kDa). M, Size marker.

Plasminogen activation by the various *Y. pseudotuberculosis* mutants

As expected, in the absence of pPla, *Y. pseudotuberculosis* strains (wild-type and the O-Ag⁻ derivative) did not exhibit any plasminogen-activation activity, at either 28 or 37 °C (Fig. 4). In contrast, the recombinant IP32953(pPla) strain was able to activate plasminogen. However, this was observed only when the bacteria were grown at 37 °C, and the activity was much lower than that observed in *Y. pestis* (Fig. 4). In the absence of O-Ag, plasminogen activation

by IP32953(pPla) O-Ag⁻ was much more efficient, and almost reached the levels detected in wild-type *Y. pestis*. This activity was observed when recombinant bacteria were grown at either 28 or 37 °C (Fig. 4). These data are in accordance with previous results indicating that expression of O-Ag prevents plasminogen activation by Pla (Kukkonen *et al.*, 2004), and they show that a mutation preventing the formation of O-Ag repeats in *Y. pseudotuberculosis* allows a Pla activity similar to that of *Y. pestis*.

α_2 AP degradation by *Y. pestis* and the various *Y. pseudotuberculosis* mutant strains

The ability of Pla to inactivate α_2 AP was evaluated by incubating the various *Y. pestis* and *Y. pseudotuberculosis* strains with α_2 AP and testing the capacity of the α_2 AP remaining in the supernatant to inhibit plasmin. In the absence of pPla, the *Y. pseudotuberculosis* supernatant did not prevent plasmin inhibition by α_2 AP, at either 28 or 37 °C (Fig. 5). In the presence of pPla, a plasmin activity was detected, indicating that α_2 AP was inactivated by IP32953(pPla). This inactivation was as efficient as that of *Y. pestis*, but only at a temperature of 37 °C (Fig. 5). In contrast, in the O-Ag mutant of *Y. pseudotuberculosis*, inactivation of α_2 AP was observed at both 28 and 37 °C, and was similar to that exhibited by *Y. pestis* (Fig. 5). These results demonstrate that, as for plasminogen activation, Pla-mediated degradation of α_2 AP is inhibited in the presence of O-Ag repeats in *Y. pseudotuberculosis*.

Virulence of the various *Y. pseudotuberculosis* mutants for mice

In order to determine whether acquisition of pPla might have an impact on *Y. pseudotuberculosis* virulence, tenfold dilutions of the wild-type strain IP32953, and of the three

Fig. 4. Plasminogen-activation activity of *Y. pestis* and *Y. pseudotuberculosis* strains grown at 28 or 37 °C. Bacteria were incubated with human plasminogen and a chromogenic plasmin substrate. Breakdown of the chromogenic substrate was measured as an A_{405} value, at time intervals of 15 min. The experiments were performed twice in duplicate and the SDs are indicated by bars. $\blacklozenge$, *Y. pestis*; $\bullet$, *Y. pseudotuberculosis* IP32953; $\circ$, IP32953(pPla); $\square$, IP32953(pPla) O-Ag⁻; $\blacksquare$, IP32953 O-Ag⁻.

Fig. 5. α_2 AP inactivation by *Y. pestis* and *Y. pseudotuberculosis* IP32953 (Y.PST) strains grown at 28 or 37 °C. Bacteria were incubated overnight with α_2 AP, and the supernatant was incubated for 90 min at 37 °C with human plasmin and the plasmin substrate. Plasmin activity was measured as breakdown of the substrate at A_{405} . The positive control for plasmin activity was incubation without bacteria and with no α_2 AP added. The positive control for α_2 AP activity was incubation without bacteria. Vertical bars represent the SDs of experiments performed twice in duplicate.

derivatives (pPla, O-Ag⁻ and pPla O-Ag⁻), were injected subcutaneously into mice in groups of 5, and mortality was recorded daily for 21 days. Although not natural for *Y. pseudotuberculosis*, the sc route of infection was used in order to mimic the *Y. pestis* mode of transmission, and determine whether acquisition of pPla facilitates *Y. pseudotuberculosis* dissemination from its site of inoculation and enhances mouse lethality. We noted that mouse lethality did not correlate with the number of bacteria when low doses of *Y. pseudotuberculosis* were inoculated (i.e. a 1000-fold increase in bacterial numbers did not lead to an increase in animal death). However, a correlation between bacterial numbers and mouse lethality was observed for bacterial doses $\geq 10^5$ c.f.u. This is in marked contrast to *Y. pestis* for which a good association between the number of bacteria injected and animal death is observed even at low doses. In order to compare the virulence of the various *Y. pseudotuberculosis* derivatives upon sc injection into mice,

bacterial inocula of $\geq 10^5$ c.f.u. were thus used. Strain IP32953(pPla) had an LD₅₀ (2.1×10^5 c.f.u.) of the same order of magnitude as that of the wild-type strain (6.1×10^5 c.f.u.), thus confirming that introduction of pPla into *Y. pseudotuberculosis* does not significantly enhance its virulence for mice. To rule out the possibility that pPla-Tmp was lost by *Y. pseudotuberculosis* during growth *in vivo*, the spleens of IP32953(pPla)-infected mice were removed and processed, and the resultant percentage of Tmp^R colonies was determined. One hundred per cent of the colonies from three different animals were Tmp^R, indicating that pPla was stably maintained during the infectious process. The O-Ag⁻ mutant strain exhibited a ≈ 10 -fold increase in LD₅₀ (5.8×10^6 c.f.u.), suggesting that O-side-chain expression plays some role in *Y. pseudotuberculosis* virulence. The IP32953(pPla) O-Ag⁻ mutant strain had the higher LD₅₀ (1.4×10^7 c.f.u.), which was ≈ 23 -fold higher than that of the wild-type strain. Analysis of the kinetics of mouse lethality also indicated that animal death was delayed for both the IP32953 O-Ag⁻ and IP32953(pPla) O-Ag⁻ mutant strains (Fig. 6). Therefore, the absence of O-Ag expression alters the virulence of *Y. pseudotuberculosis* and this defect is not compensated by the presence of pPla.

DISCUSSION

Y. pestis is a clonal species which emerged fewer than 20 000 years ago from *Y. pseudotuberculosis* (Achtman *et al.*, 1999). During its short evolution, *Y. pestis* has acquired a pathogenic potential quite exceptional in the bacterial kingdom. However, the molecular bases for this extreme pathogenicity are still not understood. One of the major genetic differences between the plague bacillus and its recent ancestor *Y. pseudotuberculosis* is the acquisition of two plasmids, pFra and pPla. While the prominent function of

Fig. 6. Kinetics of mouse lethality upon sc injection of 10^7 c.f.u. of wild-type and three derivatives of *Y. pseudotuberculosis* IP32953. Five mice were infected for each strain, and the number of mice still alive was recorded daily. ◆, IP32953; ●, IP32953(pPla); △, IP32953 O-Ag⁻; □, IP32953(pPla) O-Ag⁻.

pFra appears to be to enhance transmission of *Y. pestis* by fleas, pPla seems to play a role in the virulence of this species (Sodeinde *et al.*, 1992). Of the four proteins encoded by this plasmid, only Pla was shown to be important for *Y. pestis* virulence (Sodeinde *et al.*, 1992). The major function attributed to this outer-membrane protein is to activate plasminogen and to destroy the main control system for plasmin proteolysis. The uncontrolled proteolysis created can be targeted to the ECM and/or fibrin, potentially enhancing bacterial migration (reviewed by Korhonen *et al.*, 2004).

Whether or not acquisition of pPla during *Y. pestis* evolution has been sufficient to confer an increased pathogenicity to this species was a key question. In an attempt to answer it, Kuttyrev *et al.* (1999) introduced pPla into *Y. pseudotuberculosis*. They observed that Pla was expressed at the surface of the host strains and was processed to a smaller form designated β -Pla, as is known to be the case in *Y. pestis* (Kukkonen *et al.*, 2001; Sodeinde & Goguen, 1988). In agreement with these results, we found in this study that introduction of pPla into *Y. pseudotuberculosis* led to the synthesis of Pla, to levels similar to those of *Y. pestis*, to its export to the bacterial membrane, and to its autoprocesing. When we looked at the plasminogen activation activity of Pla, we observed that this activity was detected in cells grown at 37 °C but not in those grown at 28 °C. In contrast to Kuttyrev *et al.* (1999), we found that plasminogen activation at 37 °C was much lower than that of *Y. pestis*. These results may be explained by the recent demonstration that Pla requires rough LPS to activate plasminogen, but is inhibited in the presence of O-Ag repeats (Kukkonen *et al.*, 2004).

Y. pseudotuberculosis is known to exhibit temperature-dependent O-Ag expression (Bengoechea *et al.*, 1998). At 28 °C, O-Ag repeats are present on the conserved core oligosaccharide and could thus prevent plasminogen activation by IP32953(pPla). At 37 °C, the LPS is rough and could allow functional Pla molecules to be present on the surface of the recombinant bacterium. The decreased plasminogen activation in IP32953(pPla), as compared to *Y. pestis*, might be attributed to a reduced number, but not a complete absence, of O side chains on the LPS of *Y. pseudotuberculosis* grown at 37 °C. This hypothesis is reinforced by our observation that in *Y. pestis*, which naturally harbours a completely rough LPS because of mutations in genes involved in O-side-chain assembly (Skurnik *et al.*, 2000), plasminogen activation activity is similar in bacteria grown at 28 and 37 °C. Further demonstration that the O-Ag repeats are responsible for the absence of plasminogen activation by IP32953(pPla) grown at 28 °C, and for a low activity in cells grown at 37 °C, was obtained by introducing a mutation that abrogates the formation of O-Ag repeats (as in natural isolates of *Y. pestis*) in the chromosome of IP32953(pPla). The IP32953(pPla) O-Ag⁻ mutant strain exhibited a temperature-independent plasminogen-activation activity that was much higher than that of the parental strain IP32953(pPla), and was close to

that found in *Y. pestis*. Altogether these results demonstrate that a mutation that abrogates the synthesis of the O side chains in *Y. pseudotuberculosis* harbouring pPla allows a plasminogen-activation activity similar to that of *Y. pestis*, at both 28 and 37 °C.

In addition to increasing plasmin production by plasminogen activation, Pla also overcomes inhibition of plasmin by the serine protease inhibitor α_2 AP (Kukkonen *et al.*, 2001). In the human circulation, unbound plasmin is rapidly inactivated by plasma antiproteases, the main one being α_2 AP. We found that α_2 AP inactivation was effective in *Y. pseudotuberculosis* IP32953(pPla) grown at 37 °C, but was not detectable in bacteria grown at 28 °C. In contrast, α_2 AP degradation was similar in *Y. pestis* grown at 28 and 37 °C. These results again suggest a role for the LPS O side chains on the temperature-dependent capacity of Pla to inactivate α_2 AP in *Y. pseudotuberculosis*. The demonstration that an O-Ag⁻ derivative of IP32953(pPla) acquired the capacity to degrade α_2 AP to the same extent as *Y. pestis* when grown at either 28 or 37 °C confirmed this hypothesis.

Therefore, Pla-mediated increased production of plasmin, resulting from both plasminogen activation and α_2 AP degradation, is prevented at 28 °C in *Y. pseudotuberculosis* because of the presence of LPS O side chains, but is observable at 37 °C, a temperature at which O-Ag repeats are reduced or absent *in vitro*. However, this inhibition of O-side-chain formation at 37 °C does not appear to occur *in vivo*. This is demonstrated by the fact that during human infections with *Y. pseudotuberculosis*, high titres of antibodies directed against the O-Ag repeats of the various serotypes of this species are detected (Chung *et al.*, 1997; Schmidt, 1965; Sizaret & Mollaret, 1968; Splino *et al.*, 1969). The *in vivo* production of a smooth LPS may thus prevent the activity of Pla when a *Y. pseudotuberculosis* strain harbouring pPla infects its host. This could potentially explain why no difference in virulence was observed by Kuttyrev *et al.* (1999) between wild-type *Y. pseudotuberculosis* and smooth pPla-harboursing derivatives. We thus used an O-Ag mutant of IP32953(pPla), which was shown to exhibit plasminogen-activation and α_2 AP-degradation activities similar to those of *Y. pestis*, to compare its virulence to that of the wild-type strain and of the IP32953(pPla) and IP32953 O-Ag⁻ variants. First, our results confirmed the absence of a difference in mouse lethality between the pPla-harboursing and non-harboursing *Y. pseudotuberculosis* smooth strains. Second, they indicated that absence of O-Ag expression results in a slight decrease in *Y. pseudotuberculosis* pathogenicity. The role of LPS in *Y. pseudotuberculosis* virulence was not demonstrated until now, but was indirectly suggested by results of *in vivo* gene expression technologies (Darwin, 2004). Our data further suggest that O-side-chain production is necessary for the expression of full virulence in *Y. pseudotuberculosis*, at least by the sc route of infection. Third, our results showed that introduction of pPla into the IP32953 O-Ag⁻ variant does not increase the pathogenicity of *Y. pseudotuberculosis*. On the contrary, the

presence of pPla further decreased the virulence of the IP32953 O-Ag⁻ mutant strain and increased the time to death of the animals.

Altogether, our results indicate that *in vitro*, plasminogen-activation and α_2 AP-degradation activities can reach levels similar to those of *Y. pestis* in a *Y. pseudotuberculosis* variant harbouring pPla and lacking O-side-chain repeats. However, the efficient activity of Pla is not sufficient to confer a higher pathogenic potential to the *Y. pseudotuberculosis* host strain. The deleterious effect of a rough LPS on *Y. pseudotuberculosis* pathogenicity demonstrated in this study, may compensate the virulence-promoting effect of pPla. Furthermore, due to its protease activity, Pla may degrade proteins important for *Y. pseudotuberculosis* pathogenicity. This has been shown for YadA (Kutyrev *et al.*, 1999) and may be true for additional surface-exposed virulence factors. Although pPla may be necessary for the full virulence of most *Y. pestis* strains, this plasmid may not be sufficient by itself to explain the difference in the virulence of *Y. pestis* and *Y. pseudotuberculosis*. Additional genetic changes (mutations, deletions and/or gene acquisition) that occurred in *Y. pestis* upon its divergence from *Y. pseudotuberculosis* most likely play a critical role in the virulence of the plague bacillus. Therefore, acquisition of pPla by the *Y. pestis* ancestor may not have been sufficient to confer an immediate higher pathogenic potential to the host strain. Interplay between the acquired Pla protease and other bacterial components may have gradually led to subtle modifications of the *Y. pestis* ancestor's genetic background, and finally to the emergence of the highly pathogenic plague bacillus.

ACKNOWLEDGEMENTS

F.P. was the recipient of a grant from the French 'Ministère de la Recherche et de la Technologie'. T.K.K. was supported by the Academy of Finland (grant numbers 105824, 211300, and the Microbes and Man Research Programme). We thank Corinne Fayolle for her help during some animal experiments.

REFERENCES

- Achtman, M., Zurth, K., Morelli, C., Torrea, G., Guiyoule, A. & Carniel, E. (1999). *Yersinia pestis*, the cause of plague, is a recently emerged clone of *Yersinia pseudotuberculosis*. *Proc Natl Acad Sci U S A* **96**, 14043–14048.
- Bengoechea, J. A., Brandenburg, K., Seydel, U., Diaz, R. & Moriyon, I. (1998). *Yersinia pseudotuberculosis* and *Yersinia pestis* show increased outer membrane permeability to hydrophobic agents which correlates with lipopolysaccharide acyl-chain fluidity. *Microbiology* **144**, 1517–1526.
- Chain, P. S., Carniel, E., Larimer, F. W. & 20 other authors (2004). Insights into the evolution of *Yersinia pestis* through whole-genome comparison with *Yersinia pseudotuberculosis*. *Proc Natl Acad Sci U S A* **101**, 13826–13831.
- Chung, S.-I., Kim, W.-Y., Kim, K.-J. & Choi, C.-S. (1997). Seropositive rate to *Yersinia enterocolitica*-*pseudotuberculosis* complex among patients with acute gastroenteritis in children, 1994–1997. *J Korean Soc Microbiol* **32**, 701–715.
- Conchas, R. F. & Carniel, E. (1990). A highly efficient electroporation system for transformation of *Yersinia*. *Gene* **87**, 133–137.
- Darwin, A. (2004). Identification of *Yersinia* genes expressed during host infection. In *Yersinia, Molecular and Cellular Biology*, pp. 149–168. Edited by E. Carniel & B. J. Hinnebusch. Norfolk: Horizon Bioscience.
- Davis, K. J., Fritz, D. L., Pitt, M. L., Welkos, S. L., Worsham, P. L. & Friedlander, A. M. (1996). Pathology of experimental pneumonic plague produced by fraction 1-positive and fraction 1-negative *Yersinia pestis* in African green monkeys (*Cercopithecus aethiops*). *Arch Pathol Lab Med* **120**, 156–163.
- Derbise, A., Lesic, B., Dacheux, D., Ghigo, J. M. & Carniel, E. (2003). A rapid and simple method for inactivating chromosomal genes in *Yersinia*. *FEMS Immunol Med Microbiol* **38**, 113–116.
- Friedlander, A. M., Welkos, S. L., Worsham, P. L., Andrews, G. P., Heath, D. G., Anderson, G. W., Pitt, M. L. M., Estep, J. & Davis, K. (1995). Relationship between virulence and immunity as revealed in recent studies of the F1 capsule of *Yersinia pestis*. *Clin Infect Dis* **21**, S178–S181.
- Hinnebusch, J., Cherepanov, P., Du, Y., Rudolph, A., Dixon, J. D., Schwan, T. & Forsberg, A. (2000). Murine toxin of *Yersinia pestis* shows phospholipase D activity but is not required for virulence in mice. *Int J Med Microbiol* **290**, 483–487.
- Hinnebusch, B. J., Rudolph, A. E., Cherepanov, P., Dixon, J. E., Schwan, T. G. & Forsberg, A. (2002). Role of *Yersinia* murine toxin in survival of *Yersinia pestis* in the midgut of the flea vector. *Science* **296**, 733–735.
- Korhonen, T. K., Kukkonen, M., Virkola, R., Lång, H., Suomalainen, M., Kyllonen, P. & Läähteenmäki, K. (2004). The plasminogen activator Pla of *Yersinia pestis*: localized proteolysis and systemic spread. In *Yersinia, Molecular and Cellular Biology*, pp. 349–362. Edited by E. Carniel & B. J. Hinnebusch. Norfolk: Horizon Bioscience.
- Kukkonen, M., Läähteenmäki, K., Suomalainen, M., Kalkkinen, N., Emödy, L., Lång, H. & Korhonen, T. K. (2001). Protein regions important for plasminogen activation and inactivation of α_2 -antiplasmin in the surface protease Pla of *Yersinia pestis*. *Mol Microbiol* **40**, 1097–1111.
- Kukkonen, M., Suomalainen, M., Kyllonen, P., Läähteenmäki, K., Lång, H., Virkola, R., Helander, I. M., Holst, O. & Korhonen, T. K. (2004). Lack of O-antigen is essential for plasminogen activation by *Yersinia pestis* and *Salmonella enterica*. *Mol Microbiol* **51**, 215–225.
- Kutyrev, V. V., Filippov, A. A., Shavina, N. & Protsenko, O. A. (1989). Genetic analysis and simulation of the virulence of *Yersinia pestis*. *Mol Gen Microbiol Virusol* **8**, 42–47.
- Kutyrev, V., Mehig, R. J., Motin, V. L., Pokrovskaya, M. S., Smirnov, G. B. & Brubaker, R. R. (1999). Expression of the plague plasminogen activator in *Yersinia pseudotuberculosis* and *Escherichia coli*. *Infect Immun* **67**, 1359–1367.
- Läähteenmäki, K., Virkola, R., Saren, A., Emödy, L. & Korhonen, T. K. (1998). Expression of plasminogen activator Pla of *Yersinia pestis* enhances bacterial attachment to the mammalian extracellular matrix. *Infect Immun* **66**, 5755–5762.
- Läähteenmäki, K., Kyllonen, P., Partanen, L. & Korhonen, T. K. (2005). Antiprotease inactivation by *Salmonella enterica* released from infected macrophages. *Cell Microbiol* **7**, 529–538.
- Lesic, B. & Carniel, E. (2005). Horizontal transfer of the high-pathogenicity island of *Yersinia pseudotuberculosis*. *J Bacteriol* **187**, 3352–3358.

- Parkhill, J., Wren, B. W., Thomson, N. R. & 32 other authors (2001).** Genome sequence of *Yersinia pestis*, the causative agent of plague. *Nature* **413**, 523–527.
- Plow, E. F., Ploplis, V. A., Carmeliet, P. & Collen, D. (1999).** Plasminogen and cell migration *in vivo*. *Fibrinol Proteol* **13**, 49–53.
- Reed, L. J. & Muench, H. (1935).** A simple method of estimation fifty per cent endpoints. *Am J Hyg* **27**, 493–497.
- Rudolph, A. E., Stuckey, J. A., Zhao, Y., Matthews, H. R., Patton, W. A., Moss, J. & Dixon, J. E. (1999).** Expression, characterization, and mutagenesis of the *Yersinia pestis* murine toxin, a phospholipase D superfamily member. *J Biol Chem* **274**, 11824–11831.
- Saksela, O. (1985).** Plasminogen activation and regulation of pericellular proteolysis. *Biochim Biophys Acta* **823**, 35–65.
- Samoilova, S. V., Samoilova, L. V., Yezhov, I. N., Drozdov, I. G. & Anisimov, A. P. (1996).** Virulence of pPst(+) and pPst(-) strains of *Yersinia pestis* for guinea-pigs. *J Med Microbiol* **45**, 440–444.
- Schmidt, V. J. (1965).** Untersuchungen über den fluoreszenzserologischen Nachweis von *Pasteurella pseudotuberculosis*-Antikörpern. *Pathol Microbiol* **28**, 21–25.
- Sizaret, P. & Mollaret, H. H. (1968).** Sensibilité et spécificité comparées des réactions d'agglutination et d'immunofluorescence indirecte vis-à-vis du bacille de Malassez et Vignal "*Pasteurella pseudotuberculosis*". In *International Symposium on Pseudotuberculosis*, pp. 197–206. Edited by R. H. Regamey, A. de Barbieri, W. Hennessen, D. Ikié, F. T. Perkins & J. Ungar. Basel: S. Karger.
- Skurnik, M., Peippo, A. & Ervela, E. (2000).** Characterization of the O-antigen gene clusters of *Yersinia pseudotuberculosis* and the cryptic O-antigen gene cluster of *Yersinia pestis* shows that the plague bacillus is most closely related to and has evolved from *Yersinia pseudotuberculosis* serotype O:1b. *Mol Microbiol* **37**, 316–330.
- Sodeinde, O. A. & Goguen, J. D. (1988).** Genetic analysis of the 9·5-kilobase virulence plasmid of *Yersinia pestis*. *Infect Immun* **56**, 2743–2748.
- Sodeinde, O. A., Subrahmanyam, Y. V. B. K., Stark, K., Quan, T., Bao, Y. D. & Goguen, J. D. (1992).** A surface protease and the invasive character of plague. *Science* **258**, 1004–1007.
- Splino, M., Kyntera, F., Peychl, L. & Kotlik, J. (1969).** Ein beitrage zur serologie und epidemiologie der humanen *Pasteurella-Pseudotuberkulose*. *Pathol Anat Inst Med Fak der Karlssuniversität* **209**, 204–211.
- Welkos, S. L., Friedlander, A. M. & Davis, K. J. (1997).** Studies on the role of plasminogen activator in systemic infection by virulent *Yersinia pestis* strain C092. *Microb Pathog* **23**, 211–223.
- World Health Organization (2003).** Human plague in 2000 and 2001. *Wkly Epidemiol Rec* **78**, 130–135.