Transcriptome analysis of *Yersinia pestis* in human plasma: an approach for discovering bacterial genes involved in septicaemic plague

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Yersinia pestis is the aetiologic agent of plague. Without appropriate treatment, the pathogen rapidly causes septicaemia, the terminal and fatal phase of the disease. In order to identify bacterial genes which are essential during septicaemic plague in humans, we performed a transcriptome analysis on the fully virulent *Y. pestis* CO92 strain grown in either decomplexed human plasma or Luria–Bertani medium, incubated at either 28 or 37 °C and harvested at either the mid-exponential or the stationary growth phase. *Y. pestis* genes involved in 12 iron-acquisition systems and one iron-storage system (*bfr*, *bfd*) were specifically induced in human plasma. Of these, the *ybt* and *tonB* genes (encoding the yersiniabactin siderophore virulence factor and the siderophore transporter, respectively) were induced at 37 °C, i.e. under conditions mimicking the mammalian environment. Growth in human plasma also upregulated genes involved in the synthesis of five fimbrial-like structures (including the Psa virulence factor), and in purine/pyrimidine metabolism (the *nrd* genes). Genes known to play a role in the virulence of several bacterial pathogens (such as those encoding the Lpp lipoprotein and non-iron metal-uptake proteins) were induced in human plasma, during either the exponential or the stationary phase. Finally, 120 genes encoding proteins of unknown function were upregulated in human plasma. Eleven of these genes were specifically transcribed at 37 °C and may thus represent new virulence factors that are important during the septicaemic phase of human plague.

Received 22 January 2007

Revised 14 May 2007

Accepted 14 May 2007

INTRODUCTION

Yersinia pestis is a highly infectious, Gram-negative bacterium that causes plague. The pathogen belongs to the family *Enterobacteriaceae* and is subdivided into three classic biovars [Antiqua, Medievalis and Orientalis (Devignat, 1951)] and one additional biovar [designated

Microtus or Pestoides (Zhou et al., 2004)] which is thought to represent the ancestral *Y. pestis* branch (Achtman et al., 2004). Although plague is primarily a zoonotic disease, humans are also extremely susceptible to infection, with recent reports of about 2000 cases per year (<http://www.who.int/mediacentre/factsheets/fs267/en/>). Three main clinical forms are designated according to the route of infection (Dennis et al., 1999). Bubonic plague is the most common form and results from the bite of an infected flea. Pneumonic plague is less common but is considered to be highly contagious, since it is transmitted from human to human by aerosols; the incubation period is very short (1–3 days) and death ensues almost invariably if appropriate antibiotic therapy is not initiated within 18–24 h of disease onset. Septicaemic plague is the least frequent and most fulminating, fatal form of plague. It results from direct penetration of

Abbreviation: QRT-PCR, quantitative real-time PCR.

A supplementary figure showing QRT-PCR confirmation of transcriptome results, and four supplementary tables listing the fold-changes due to medium, temperature and growth phase, and the 30 most highly expressed *Y. pestis* chromosomal genes in human plasma, are available with the online version of this paper.

The array data discussed in this publication have been deposited in the Genoscript database (<http://genoscript.pasteur.fr>) and are accessible as experiment 'Human plasma *Y. Pestis*'.

the bacterium into the bloodstream through skin lesions or via the conjunctiva. In all three forms, the terminal phase of plague is characterized by bacterial dissemination into the bloodstream, leading to rapid death of the host.

The bacterial factors required during the septicaemic phase of plague are largely unknown. Certain virulence factors identified in *Y. pestis* appear to be more important in the initial phase of the infection (enabling the bacterium to disseminate into its host) than in the septicaemic phase. This is the case for the Pla protease, a cell-surface plasminogen activator which is essential for the development of bubonic plague via peripheral routes of infection, but which has little impact on septicaemic plague (i.e. when the bacteria are delivered intravenously or via a flea bite directly into the bloodstream) (Sebbane *et al.*, 2006a; Sodeinde *et al.*, 1992). Similarly, even though the yersiniabactin-dependent iron-uptake system is required for development of a fatal *Y. pestis* infection when bacteria are injected subcutaneously, the system is completely non-essential following intravenous infection (Bearden *et al.*, 1997; Bearden & Perry, 1999; Une & Brubaker, 1984). A few known *Y. pestis* virulence factors have been shown to be required for development of the disease when bacteria are directly inoculated into the bloodstream. This is true for the pCD1 plasmid, a major virulence factor that delivers toxic proteins into host cells and which is essential for triggering plague when the bacteria are delivered peripherally or directly into the bloodstream (Brubaker, 2000). Likewise, the Yfe iron-acquisition system seems to play a key role after either subcutaneous or intravenous infection in mice (Bearden & Perry, 1999). Lastly, the PsaA fimbria (an antiphagocytic factor also known as pH 6 antigen) is involved in plague disease when bacteria are injected intravenously (Lindler *et al.*, 1990).

Since little is known about the *Y. pestis* factors that are specifically turned on during the septicaemic phase of the disease, we decided to analyse the overall transcriptional response of *Y. pestis* during growth in conditions resembling plague septicaemia. For this purpose, we prepared whole-genome macroarrays of fully virulent *Y. pestis* CO92 biovar Orientalis (Parkhill *et al.*, 2001). This strain was grown in human plasma or Luria-Bertani (LB) medium and at either 37 or 28 °C. Since the physiological status of *Y. pestis* in human blood is not known, transcription profiling of both mid-exponential and stationary-phase bacteria was performed. Hence, we carried out transcriptome analysis under a total of eight different growth conditions (examining three variables: medium, temperature and growth phase). We first identified genes induced in human plasma and then focused on those specifically induced at 37 °C or showing growth-phase-dependent transcriptional regulation.

METHODS

Preparation of whole-genome macroarrays. Pairs of specific primers were designed for each of the 4221 ORFs present on the *Y.*

pestis CO92 chromosome and the three plasmids, using the CAAT-Box and Primer3 software packages (Frangoul *et al.*, 2004; Parkhill *et al.*, 2001). Primers were chosen in order to amplify 400–500 bp fragments with a melting temperature of 55 °C. Briefly, the CAAT-Box software automatically selected the part of a gene which presented the least similarity to the rest of the genome and then checked that the primer pairs (provided by Primer3 software) were unlikely to produce non-specific amplifications. Next, to ensure specificity, the theoretical PCR products were compared with the genome by BLASTN. A total of 4126 primer pairs were designed and synthesized. Amplification reactions were performed by Eurogentec and were successful for 97% of primer pairs. PCR products were spotted on nylon membranes (Genetix) previously soaked in 10 mM Tris, 1 mM EDTA, pH 7.6, using a QPix robot (Genetix). Genomic DNA from *Y. pestis* CO92 and a range of amounts (10–100 ng) of luciferase DNA (Promega) were spotted as positive controls. Salmon sperm DNA and DNA-free samples were spotted as negative controls. Membrane-spotted DNA was denatured for 15 min in 0.5 M NaOH, 1.5 M NaCl, washed three times with water and then dried on Whatman paper. Successful spotting of DNA samples on nylon membranes was ensured by performing hybridization with ³²P-labelled genomic DNA from *Y. pestis* CO92. Membranes were stored at –20 °C until use.

Bacterial strain and media. The fully virulent *Yersinia pestis* strain CO92 (biovar Orientalis) was grown in either LB medium or human plasma. A pool of 56 human plasma samples (Etablissement Français du Sang) was aliquoted and conserved at –20 °C. Before use, human plasma was decomplexed by incubation at 56 °C for 30 min and was then partially clarified by centrifugation at 5000 g for 10 min.

Bacterial growth. For each experiment, *Y. pestis* CO92 was taken from the –80 °C stock, streaked on LB plates containing 20 µg haemin ml^{–1} and incubated for 48 h at 28 °C. An inoculum was prepared by suspending bacteria from the plate into 0.15 M NaCl, followed by dilution to OD₆₀₀ 0.02 in 40 ml (2 × 10⁷ bacteria ml^{–1}) culture medium pre-heated to 37 or 28 °C. Cultures were performed at either 28 or 37 °C with shaking. Growth was monitored by measuring OD₆₀₀. In view of the high optical density of human plasma at 600 nm, samples were diluted twofold in 0.15 M NaCl prior to reading the OD₆₀₀. The OD₆₀₀ values of infected plasma were determined by subtracting the OD₆₀₀ value of uninfected plasma.

Isolation of total RNA. Either 10 ml *Y. pestis* grown in LB or 40 ml *Y. pestis* grown in human plasma was centrifuged at 6000 g for 5 min at 4 °C. Pellets were promptly suspended in 1 ml RNAwiz (Ambion) and immediately stored at –80 °C. The defrosted lysate was treated with 200 µl chloroform, vigorously shaken for 20 s and incubated at room temperature for 10 min. The aqueous phase was recovered after centrifugation at 4 °C (12 000 g for 10 min) and diluted with 450 µl H₂O. RNA was precipitated by addition of 18 µl nuclease-free glycogen (5 mg ml^{–1}; Ambion) and 900 µl 2-propanol, then pelleted by centrifugation at 12 000 g for 30 min at 4 °C. Pellets were rinsed with cold 70% ethanol, dried and suspended in 50 µl water. DNA was removed from the RNA samples by adding 4 U DNase, as described in the instructions for use of the DNA-free kit (Ambion). Total RNA was quantified by A₂₆₀. RNA purity and quality were checked using an Agilent 2100 bioanalyser. Purified RNA samples were aliquoted and stored at –80 °C until use.

Probe synthesis. Radioactive cDNA probes were prepared from RNA by incorporation of [³²P]dCTP during first-strand cDNA synthesis with reverse transcriptase. For all experiments, the same mix of *Yersinia*-specific reverse primers (0.1 pM) containing 0.1 pM luciferase primer (Eurogentec) was used. Labelling was performed in a final volume of 45 µl containing 10 µg RNA, 0.5 ng luciferase RNA (Promega), 0.35 pmol *Yersinia*-specific primers, 9 µl 4 × incubation

buffer (Roche), 10 mmol dATP, 10 mmol dTTP, 10 mmol dGTP, 0.1 mmol dCTP, 50 μ Ci (1.85 MBq) [α - 32 P]dCTP (Amersham Biosciences) and 50 U AMV reverse transcriptase (Roche). In the first step, primer hybridization was performed using a 22 μ l mixture of total *Y. pestis* RNA, luciferase RNA and the primers. After incubation at 90 °C for 2 min, the heating block was placed on the bench until the temperature dropped to 42 °C. In the second step, labelling was performed by addition of dNTPs, reverse transcriptase, [32 P]dCTP and reverse transcriptase buffer, followed by incubation at 42 °C for 3 h. Unincorporated nucleotides were removed from the labelled cDNA by using the DyeEx 2.0 spin kit (Qiagen).

Macroarray hybridization. Membranes were hybridized in a random order so as to avoid artefacts due to technical variations encountered while performing the series of 24 hybridizations. Macroarrays were soaked in $2 \times$ SSPE buffer ($1 \times$ SSPE is 0.18 M NaCl, 10 mM NaH₂PO₄, 1 mM EDTA, pH 7.7) and pre-hybridized for 1 h in the hybridization solution ($5 \times$ SSPE, 2% SDS, $1 \times$ Denhardt's reagent, 0.1 mg ml⁻¹ salmon sperm DNA) at 65 °C in roller bottles. Hybridization with the entire, spin-purified, denatured cDNA probe sample was carried out for 20 h at 65 °C in 5 ml fresh hybridization solution. Membranes were washed three times in washing buffer ($0.5 \times$ SSPE, 0.2% SDS) at room temperature for 3 min, and three times with pre-heated washing buffer at 65 °C for 20 min. Macroarrays were sealed in 25 μ m-thick plastic sheets and then exposed to a phosphor screen (Molecular Dynamics) for 20 h. Membranes were scanned using the STORM 860 phosphorimager (Amersham Biosciences).

Quantitative real-time PCR (QRT-PCR). cDNA was synthesized using 1 μ g total RNA and the Transcriptor First Strand cDNA Synthesis kit (Roche). Subsequently, real-time PCR was conducted using the LightCycler 2.0 System (Roche) according to the manufacturer's instructions. Fluorescent PCR products were generated from cDNA using the LightCycler FastStart DNA Master^{PLUS} SYBR Green I kit (Roche) and specific primers for each gene which were originally designed for macroarray preparation. Relative quantification was determined using YPO3356 (*nlpD* gene) as a standard, and was corrected using the PCR efficiency of each primer set.

Data analysis. ArrayVision software (Imaging Research) was used to quantify hybridization intensities. The intensity data were log-transformed and normalized using a simple median normalization method. Next, an ANOVA was performed first on the whole dataset and then independently for each gene, with the three variables (medium, temperature and growth phase) as fixed effects. All statistical analyses were carried out using the SAS software (SAS Institute). Three *P* values were calculated per gene. Since the *P* value calculation is based on a null hypothesis that states that differential expression is not genuine, low *P* values indicate that the difference in expression is unlikely to have occurred by chance, whereas high *P* values indicate that the differential expression is likely to be false. In other words, low *P* values correspond to specific up- and down-regulation, whereas high *P* values correspond to an absence of differential expression. Transcriptional regulation and constitutive expression were considered to be statistically significant when the *P* value was below 0.05 and above 0.5, respectively.

RESULTS AND DISCUSSION

Experimental design

In order to identify *Y. pestis* genes which are likely to be specifically induced during the septicaemic phase of

infection in humans, we performed comparative expression profiling of strain CO92 grown in human plasma or in the rich artificial medium LB. Human plasma and LB cultures were carried out either at the host temperature (37 °C) or at the optimal *in vitro* growth temperature for *Yersinia* (28 °C). Since the physiological status of *Y. pestis* during septicaemia is not known, bacteria were harvested during both the exponential phase (6 h of incubation) and the stationary phase (24 h of incubation). Therefore, eight growth conditions in all, including three variables (medium, temperature and growth phase), were studied. For each growth condition, three different mRNA preparations from independent cultures were used for cDNA synthesis and subsequent macroarray hybridization.

Growth of *Y. pestis* in human plasma

In a preliminary test, viable bacterial counts and OD₆₀₀ measurements were performed in parallel in order to evaluate *Y. pestis* growth in human plasma. Since both datasets gave similar results, bacterial growth was subsequently monitored using OD₆₀₀ measurements only. Given that maximal reproducibility between biological replicates is needed for reliable transcriptome analysis, *Y. pestis* culture conditions in human plasma were precisely set up. Growth of strain CO92 in human plasma batch cultures was compared before and after decomplexation. Prior to decomplexation, *Y. pestis* was able to multiply in human plasma, confirming that this pathogen is resistant to the bactericidal effect of complement (Porat *et al.*, 1995; Sodeinde *et al.*, 1992). However, the growth was characterized by high inter-experiment variation in the duration of the lag phase. When human plasma was decomplexed at 56 °C for 30 min, a short, reproducible lag phase (followed by reproducible growth curves with typical exponential and stationary phases) was obtained. Hence, human plasma was always decomplexed before use in *Y. pestis* batch cultures.

Growth curves of *Y. pestis* in decomplexed human plasma or LB were compared at 28 and 37 °C. As has been previously established (Brubaker, 2000), *Y. pestis* grew slightly more slowly in LB at 37 °C than at 28 °C (doubling time 1 h 55 min and 1 h 23 min at 37 and 28 °C respectively), reflecting physiological changes that occur between room and host temperatures. However, in decomplexed human plasma, *Y. pestis* grew notably faster at 37 °C than at 28 °C (doubling time of 1 h 42 min and 3 h 8 min at 37 and 28 °C, respectively). This suggests that under *in vivo* conditions, the complex network of temperature regulation might differ from that identified *in vitro*.

Macroarray analysis

An overall analysis of variance indicated that growth phase was the factor that introduced the most variation. The variance due to growth phase was 20-fold higher than that

due to the medium and sixfold higher than that due to temperature. This result is consistent with the tremendous changes in gene expression that reflect physiological adaptation when moving from the exponential phase into the stationary phase. More genes were regulated by the medium (715) than by temperature (461), whereas the variance due to the medium was at least threefold lower than that due to temperature. These results suggest that the medium regulates more genes than does temperature, but that it causes weaker transcriptional changes per gene.

A total of 1615 genes were found to be significantly up- or downregulated by at least one biological factor. Transcriptional fold-changes were calculated from pairwise comparisons of the conditions (human plasma vs LB; 37 vs 28 °C; mid-exponential phase vs stationary phase) using normalized intensities and are provided as supplementary data (see Supplementary Tables S1, S2 and S3, respectively, available with the online version of this paper). In order to validate the macroarray results, real-time PCR quantifications were performed on a subset of genes which exhibited differential transcription under specific conditions. As shown in Supplementary Fig. S1, both methods showed similar up- and downregulation of the genes studied.

Most of the genes (1124 out of 1615) were affected by only one factor: 344 by the medium, 216 by temperature and 564 by growth phase. A fair number of genes (420) were regulated by two factors: 58 by the medium and temperature, 245 by the medium and growth phase, and 117 by temperature and growth phase. Very few genes (71) were regulated by all three factors.

Genes which are transcriptionally induced in human plasma

Of the 315 genes induced in human plasma, a large number encode proteins of unknown (120) or miscellaneous (73) function (Fig. 1). The next most numerous class corresponds

to genes encoding proteins involved in iron metabolism (42 genes). Forty genes are involved in the synthesis of eleven putative inorganic iron or haem transport systems (Table 1), and two genes (*bfr* and *bfd*) are involved in iron storage. This is consistent with the low-iron environment encountered in plasma. Indeed, blood contains $<10^{-18}$ M extracellular free iron, which is far below the concentration necessary for bacterial growth (at least 10^{-8} M; Stanier *et al.*, 1986). Hence, bacterial survival and multiplication in such a low-iron milieu depend on the expression of iron uptake systems. Siderophores are sophisticated, high-affinity iron chelators which can capture iron molecules bound to host proteins and deliver them to the bacterium. Once inside the bacterial cell, the iron is removed from the complex and used in various metabolic pathways. In *Y. pestis*, most of the genes involved in synthesis of the yersiniabactin siderophore (Ybt) displayed transcriptional induction in human plasma and at 37 °C (Table 1), indicative of a role for yersiniabactin in the septicaemic phase of plague in humans. Interestingly, transcription of *ybtA* (which encodes the AraC-like transcriptional activator of most *ybt* genes) was induced during the stationary phase (Table 1). This suggests that a signal for yersiniabactin synthesis is specifically produced during this growth phase. The ExbBD and TonB proteins form an inner-membrane complex which is involved in importing essential micronutrients across the outer membrane. Transcription of *exbBD* genes was induced in human plasma, and *tonB* transcription was upregulated both in human plasma and at 37 °C, the same transcriptional profile as that of the *ybt* genes. This matches the absolute requirement for TonB for transport of the iron-yersiniabactin complex into *Y. pestis* (Perry *et al.*, 2003). The *Y. pestis* chromosome bears a gene cluster which potentially encodes a second siderophore, named Ysu. Transcription of several *ysu* genes was induced in human plasma (Table 1), suggesting that the Ysu siderophore is

Fig. 1. Number of human plasma-induced genes per functional class, as defined in the *Y. pestis* CO92 genome annotation.

Table 1. Genes involved in iron- and haem-uptake systems, and which are significantly regulated by one of the three factors tested

Ratios (*R*) were calculated by pairwise comparison of growth conditions: human plasma vs rich medium (HP:LB), mid-exponential phase vs stationary phase (expo:stat), and 37 vs 28 °C (37:28). Absence of a value indicates that the difference was not significant ($P \geq 0.05$).

Gene ID	Name	Function	<i>R</i> (HP:LB)	<i>R</i> (expo:stat)	<i>R</i> (37:28)
Ybt siderophore iron-transport system					
YPO1906	<i>psn</i>	Pesticin/yersiniabactin receptor protein	2.5		2
YPO1907	<i>ybtE</i>	Yersiniabactin siderophore biosynthetic protein	2		1.5
YPO1908	<i>ybtT</i>	Yersiniabactin biosynthetic protein YbtT	3		
YPO1909	<i>ybtU</i>	Yersiniabactin biosynthetic protein YbtU	2.9		1.8
YPO1910	<i>irp1</i>	Yersiniabactin biosynthetic protein			1.7
YPO1911	<i>irp2</i>	Yersiniabactin biosynthetic protein	7.1	−1.4	2.5
YPO1912	<i>ybtA</i>	Transcriptional regulator YbtA		−1.7	1.5
YPO1913	<i>ybtP</i>	Inner-membrane lipoprotein ABC transporter	4		
YPO1914	<i>ybtQ</i>	Inner-membrane ABC transporter YbtQ	1.8		
YPO1915	<i>ybtX</i>	Putative signal transducer	2.9		2.1
YPO1916	<i>ybtS</i>	Putative salicylate synthetase	4.2		2.3
ExbBD–TonB inner-membrane complex					
YPO0682	<i>exbB</i>	TonB-mediated protein	3		
YPO0683	<i>exbD</i>	TonB-mediated protein	3.6	2.5	
YPO2193	<i>tonB</i>	Energy transducer and iron-uptake-related protein	2.6		1.9
Putative Ysu siderophore iron-transport system					
YPO1528	<i>ysuF</i>	Putative ferric iron reductase	3.1	1.7	
YPO1529	<i>ysuJ</i>	Putative decarboxylase		1.8	
YPO1530	<i>alcA/ysuI</i>	Putative siderophore biosynthetic enzyme			−1.8
YPO1532	<i>ysuG</i>	Putative siderophore biosynthetic enzyme	1.8	1.5	
YPO1536	<i>ysuA</i>	Putative periplasmic binding protein	2.2		
YPO1537	<i>ysuR</i>	Putative iron-siderophore receptor	3.3		
YPO1538	<i>ysuE</i>	Putative siderophore biosynthetic enzyme	6.1	2.5	
Yfe iron ABC transporter					
YPO2439	<i>yfeA</i>	Solute-binding protein	10.2		
YPO2440	<i>yfeB</i>	ATP-binding transport protein	2.8		
YPO2441	<i>yfeC</i>	Chelated iron transport system membrane protein	1.8	1.8	
YPO2445	<i>yfeE</i>	Internal membrane protein	2.1		
Yiu iron ABC transporter					
YPO1310	<i>yiuA</i>	Putative periplasmic solute-binding protein	1.7	2.1	
YPO1312	<i>yiuC</i>	Putative ATP-binding subunit	2		
YPO1313	<i>yiuR</i>	Putative outer-membrane protein	1.5		
Sfu/Yfu iron ABC transporter					
YPO2958	<i>sfuA/yfuA</i>	Iron(III)-binding periplasmic protein	3	1.7	
YPO2960	<i>sfuC/yfuC</i>	Iron(III)-transport ATP-binding protein	1.8		
Putative Fit iron ABC transporter					
YPO4022		Putative iron-transport protein	2.2	1.9	
YPO4023		Putative iron-transport permease		1.9	
YPO4025		Putative ATP-binding protein	1.4	1.6	
Hmu haem transporter					
YPO0279	<i>hmuV</i>	Haemin-transport system ATP-binding protein	1.5		
YPO0281	<i>hmuT</i>	Haemin-binding periplasmic protein	2.1		
YPO0282	<i>hmuS</i>	Haemin-transport protein	2		
YPO0283	<i>hmuR</i>	Haemin receptor precursor	2		
Has haemophore system					
YPO3922	<i>hasA</i>	Haemophore HasA	1.8		
YPO0663	<i>hasF</i>	ABC transporter outer-membrane component		2.1	
Feo ferrous iron transporter					
YPO0131	<i>feoC</i>	Putative ferrous iron transport protein C	2		−1.9
Ferric hydroxamate iron-transport systems					
YPO0989	<i>iucA*</i>	Putative siderophore biosynthesis protein IucA	2.1		
YPO0993	<i>iucD</i>	Putative siderophore biosynthesis protein IucD	−1.6		
YPO1753	<i>fcuA</i>	Ferrichrome receptor protein	1.7	1.5	

Table 1. cont.

Gene ID	Name	Function	R (HP:LB)	R (expo:stat)	R (37:28)
YPO3391	<i>fhuD</i>	Ferrichrome-binding periplasmic protein precursor		1.9	
		Putative Ynp non-ribosomal peptide synthesis system			
YPO0771		ABC transporter transmembrane protein			2
YPO1011		Putative TonB-dependent outer-membrane receptor	1.8		
YPO1012	*	Putative peptide/polyketide synthase subunit	1.8		
		Other iron-transport systems			
YPO0164		Putative iron complex outer-membrane receptor protein	−1.5		
YPO0701	<i>fepB</i>	Putative siderophore enterobactin-binding periplasmic protein		−1.5	
YPO1854		Putative high-affinity iron transporter	1.9	1.9	
YPO3050		Putative ABC transporter, periplasmic iron-binding protein	−2.7	1.9	−2
YPO3340	*	Putative exogenous ferric siderophore receptor	5		

*Pseudogene.

indeed produced and may thus participate in iron uptake in plasma. In addition to the siderophores, the *Y. pestis* chromosome also harbours several gene clusters encoding ABC transporters for inorganic iron uptake (Perry & Fetherston, 2004). The *yfeA* gene (encoding the solute-binding protein of the Yfe iron ABC transporter) was the most strongly upregulated gene (10.2-fold) in human plasma for the entire genome (Table 1, Supplementary Table S1). All other *yfe* genes were also induced in human plasma (Table 1), suggesting that the Yfe iron-acquisition function plays a role during the septicaemic phase of plague. This correlates with the observation that in a Ybt defective strain, the Yfe system is essential for virulence when *Y. pestis* is intravenously injected into mice (Bearden & Perry, 1999). Transcription of the *yfeABCE* genes was similar at 28 and 37 °C ($P>0.5$), indicating that these genes were not temperature-regulated. Growth in human plasma also stimulated transcription of genes encoding two other ABC iron transporters, the Yiu and Sfu/Yfu systems (Table 1). Both systems are known to be functional in *Y. pestis* (Kirillina *et al.*, 2006). However, iron uptake by the Yfu or Yiu system was observed only when the Ybt–Yfe and Ybt–Yfe–Yfu systems, respectively, were mutated. Iron can also be acquired from an organic source such as haem and haem-containing proteins. In *Y. pestis*, transcription of the *hmuVTSR* genes (encoding the Hmu haem transporter) was induced in human plasma (Table 1). This fits with the observation that Hmu-mediated iron uptake is functional in *Y. pestis* (Hornung *et al.*, 1996). Although the iron-acquisition function of the other five systems is putative, their transcriptional induction in human plasma suggests that they are synthesized and that they participate in iron uptake.

Eleven genes involved in the synthesis of surface structures were induced in human plasma (Fig. 1). The PsaA fimbria (also known as pH 6 antigen) is encoded by the *psaABCEF* cluster. Growth in human plasma upregulated transcription of *psaE* and *psaF* (Supplementary Table S1),

suggesting a role for Psa in the septicaemic phase of plague. Indeed, mutation of *psaE* or *psaA* attenuates *Y. pestis* virulence after intravenous infection of mice (Lindler *et al.*, 1990). Eight other gene clusters encoding putative fimbrial structures have been identified on the *Y. pestis* chromosome (Parkhill *et al.*, 2001). Genes involved in the synthesis of four of these (YPO0698–0700, YPO1695–1700, YPO1918–1925 and YPO2940–2945) were induced in human plasma (Supplementary Table S1). Taken as a whole, our results indicate that these four fimbrial structures may (like Psa) exert a function during plague septicaemia.

Genes which are transcriptionally induced in human plasma at 37 °C

In order to identify the genes which are most likely to play a role in the septicaemic phase of plague, we focused our analysis on those induced both in human plasma and at 37 °C. In addition to the seven genes involved in yersiniabactin synthesis and transport (Table 1), 23 other genes were induced under the specified conditions (Table 2). Almost half of these [including YPO1649 and YPO3527, which were respectively the fourth and fifth most highly expressed genes in human plasma (Supplementary Table S4)] encoded proteins of unknown function. The hypothetical protein encoded by YPO1516 may have a general role in plague, since it was induced not only in human plasma at 37 °C but also *in vivo* during bubonic plague in rats and during pneumonic plague in mice (Lathem *et al.*, 2005; Sebbane *et al.*, 2006b).

Two genes known to participate in *Y. pestis* virulence (*sydH* and *virG*) were induced in human plasma and at 37 °C (Table 2). Both genes are borne on the pCD1 plasmid, which is essential for virulence in the three pathogenic *Yersinia* species (*Y. pestis*, *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*) by mediating resistance to the host innate immune system (Marenne *et al.*, 2004). This virulence results from the injection of Yop effector proteins into host cells by a

type III secretion system called Ysc. Our transcriptome analysis showed that, in contrast to *syncH* and *virG*, the pCD1-borne genes *lcrG*, *yadA*, *yopBDMT* and *yscBGHIJLNOPY* were constitutively expressed in human plasma and LB. In addition, 45 (including the *ysc-yop* virulon) out of the 57 pCD1 genes spotted on the macroarrays were induced at 37 °C (Supplementary Table S2). A similar result has been observed in *Y. pestis* strain KIM5 D27 (biovar Medievalis) and strain 201 (biovar Microtus) (Han *et al.*, 2004; Motin *et al.*, 2004), which is consistent with the induction of Yop secretion on shifting from 26 to 37 °C in a calcium-free medium (Marenne *et al.*, 2004).

Lastly, nine genes encoding metabolic functions were induced in human plasma at 37 °C (Table 2). These metabolic functions include: intracellular sulphur oxidation (YPO0198 and YPO0199), biosynthesis of iron-sulphur proteins (YPO2404 of the HesB family), vitamin synthesis (*thiC* and YPO1603), carbohydrate metabolism (*glgX* and YPO3090) and nucleotide metabolism (*nrdI*). Interestingly, transcription of the other three genes composing the *nrdHIEF* ribonucleotide reductase operon was strongly

induced (9-, 2.1- and 3.4-fold) in human plasma. Indeed, after *yfeA*, *nrdE* and *nrdI* exhibited the strongest upregulation values (Supplementary Table S1). Additionally, the *Y. pestis nrdHIEF* genes have also been found to be upregulated in the bubo during rat infection (Sebbane *et al.*, 2006b). Together, these results suggest that purine/pyrimidine metabolism is essential for *Y. pestis* survival and/or growth in the mammalian host, and support the demonstration that purine metabolism is necessary for *Y. pestis* virulence (Munier-Lehmann *et al.*, 2003).

Growth-phase-dependent transcriptional regulation of genes induced in human plasma

The physiological status of *Y. pestis* during infection is not known. A recent transcriptome analysis suggests that bacteria in the bubo are in a similar state to that observed during the stationary phase *in vitro* (Sebbane *et al.*, 2006b). However, the bacteria proliferate rapidly during other stages of the infection process, which suggests that they are in an exponential growth phase. In order to identify the

Table 2. Genes not involved in iron acquisition and exhibiting transcriptional induction in human plasma at 37 °C

Ratios (*R*) were calculated by pairwise comparison of growth conditions: human plasma vs rich medium (HP:LB), mid-exponential phase vs stationary phase (expo:stat), and 37 vs 28 °C (37:28). Absence of a value indicates that the difference was not significant ($P \geq 0.05$).

Gene ID	Name	Function	<i>R</i> (HP:LB)	<i>R</i> (expo:stat)	<i>R</i> (37:28)
Involved in virulence					
YPCD1.95c	<i>syncH</i>	Secretion chaperone for YopH	2		8.3
YPCD1.48	<i>virG</i>	Yop secretion and targeting control lipoprotein	1.5		3.3
Involved in metabolism					
YPO0198		Putative intracellular sulphur oxidation protein	2.6	−1.7	2.6
YPO0199		Putative intracellular sulphur oxidation protein	1.8	−1.4	1.7
YPO1462		Putative acyl carrier protein	1.6		1.5
YPO1603	<i>pabC</i>	4-Amino-4-deoxychorismate lyase	1.8	−1.4	1.4
YPO2404		Conserved hypothetical protein (HesB domain)	3.1	2	1.7
YPO2650	<i>nrdI</i>	NrdI protein homologue	8.8		2
YPO3090	<i>ushA*</i>	UDP-sugar hydrolase	1.8	−1.5	1.7
YPO3739	<i>thiC</i>	Thiamine biosynthesis protein ThiC	2		2
YPO3941	<i>glgX</i>	Putative α -amylase	1.6		1.7
Miscellaneous					
YPO4051	<i>tnpA</i>	Transposase (partial)	1.4		1.7
Unknown					
YPO0445		Conserved hypothetical protein	1.4		1.5
YPO1516		Hypothetical protein	1.7		1.5
YPO1649		Hypothetical protein	2.7		1.8
YPO2276A		Putative phage-related membrane protein	1.4		1.7
YPO2605		Conserved hypothetical protein	1.4		1.5
YPO2606		Conserved hypothetical protein	2.7	−1.6	2
YPO2700		Conserved hypothetical protein	1.5		1.5
YPO3518		Conserved putative exported protein	2.2		2.1
YPO3527		Conserved hypothetical protein	3.6	−5.1	1.6
YPO4064		Hypothetical protein	2.7		2
YPO4086		Putative lipoprotein	1.8		2.4

*Pseudogene.

Table 3. Genes showing transcriptional induction in human plasma during exponential phase

Ratios (*R*) were calculated by pairwise comparison of growth conditions: human plasma vs rich medium (HP:LB), mid-exponential phase vs stationary phase (expo:stat), and 37 vs 28 °C (37:28). Absence of a value indicates that the difference was not significant ($P \geq 0.05$).

Gene ID	Name	Function	<i>R</i> (HP:LB)	<i>R</i> (expo:stat)	<i>R</i> (37:28)
Iron metabolism					
YPO0683	<i>exbD</i>	TonB-mediated protein	3.6	2.5	
YPO1310	<i>yiuA</i>	Putative periplasmic solute-binding protein	1.7	2.1	
YPO1528	<i>ysuF</i>	Putative ferric iron reductase	3.1	1.7	
YPO1532	<i>ysuG</i>	Putative siderophore biosynthetic enzyme	1.8	1.5	
YPO1538	<i>ysuE</i>	Putative siderophore biosynthetic enzyme	6.1	2.5	
YPO1753	<i>fcuA</i>	Ferrichrome receptor protein	1.7	1.5	
YPO1854		Putative high-affinity iron transporter	1.9	1.9	
YPO2441	<i>yfeC</i>	Chelated iron transport system membrane protein	1.8	1.8	
YPO2958	<i>sfuA/yfuA</i>	Iron(III)-binding periplasmic protein	3	1.7	
YPO4022		Putative iron-transport protein	2.2	1.9	
YPO4025		Putative iron ABC transporter, ATP-binding protein	1.4	1.6	
Stress-responsive proteins					
YPO0176		OsmC family protein	2	2.4	
YPO2342	<i>tpx</i>	Thiol peroxidase	1.8	2	−1.8
YPO4061	<i>sodA</i>	Superoxide dismutase [Mn]	1.8	3.5	
Carbohydrate metabolism					
YPO1133	<i>gpmA</i>	Phosphoglycerate mutase 1	3.7	3.1	
YPO1608	<i>ptsG</i>	Phosphotransferase system (PTS), glucose-specific IIBC component	1.7	1.7	
YPO1758	<i>manX</i>	PTS, mannose-specific IIAB component	3.2	1.8	
YPO2994	<i>ptsI</i>	PTS, enzyme I component	1.4	2.1	
YPO3718	<i>pgi</i>	Glucose-6-phosphate isomerase	1.5	1.7	
Amino acid metabolism					
YPO0914	<i>serA</i>	D-3-Phosphoglycerate dehydrogenase	1.5	1.7	
YPO1193		Putative pyridoxal-dependent decarboxylase	1.7	1.5	−1.8
YPO3727	<i>metA</i>	Homoserine O-succinyltransferase	1.5	1.8	
Vitamin metabolism					
YPO0144		Putative hydrolase	1.9	2.1	
YPO0287		Putative methylenetetrahydrofolate reductase	3.6	8.1	−3.8
YPO2214	<i>btuR</i>	Cob(I)alamin adenosyltransferase	1.7	2	
Folding					
YPO0127		Thioredoxin-like protein	3.7	2	
YPO0195	<i>fkpA</i>	Peptidyl-prolyl <i>cis-trans</i> isomerase	1.7	1.9	
Outer-membrane proteins					
YPO1684	*	Putative surface protein	2.1	4.1	−2.6
YPO1967	<i>nmpC/phmA*</i>	Outer-membrane protein	1.8	1.7	
YPO2394	<i>lpp</i>	Major outer-membrane lipoprotein	2	4	
Regulator					
YPO3545		LysR-family transcriptional regulatory protein	1.7	1.4	
Miscellaneous					
YPO0027		Putative phosphatase	1.8	5.5	−3.1
YPO2180	<i>adhE</i>	Aldehyde-alcohol dehydrogenase	3.4	1.6	
YPO2367	<i>gst</i>	Glutathione S-transferase	2.1	1.8	
YPO2404		Conserved hypothetical protein (HesB domain)	3.1	2	1.7
YPO3300	<i>luxS</i>	Autoinducer-2 production protein	1.9	2.6	−1.9
YPO3223	<i>crl</i>	Curlin genes regulatory protein	1.6	3.2	−1.6
IS element					
†		Transposase for insertion sequence IS1661	1.5	2.1	
Unknown					
YPO0100		Hypothetical protein	1.9	3	−2.2
YPO0102		Putative exported protein	1.8	4.2	−3.2
YPO0337		Putative exported protein	2	1.5	
YPO0457	<i>creA</i>	Putative exported (periplasmic) protein	2.3	3.7	−2.3

Table 3. cont.

Gene ID	Name	Function	R (HP:LB)	R (expo:stat)	R (37:28)
YPO0498		Hypothetical protein	3.7	13.9	-8.8
YPO0499		Hypothetical protein	2.4	14.3	-12.7
YPO0500		Conserved hypothetical protein	2.3	12.2	-8.2
YPO0502		Conserved hypothetical protein	1.9	20.2	-36.9
YPO0510		Hypothetical protein	1.5	2.8	-2.4
YPO0511		Hypothetical protein	1.5	1.7	
YPO0657		Hypothetical protein	2	4	
YPO1243		Hypothetical protein	1.8	1.7	-1.9
YPO1527		Putative membrane protein	1.4	1.6	-1.6
YPO1588		Conserved hypothetical protein	2.1	1.5	-1.8
YPO2226		Hypothetical protein	1.8	2	-1.6
YPO2531	<i>elaB</i>	Putative membrane protein	3.7	2	-1.6
YPO3336		Conserved hypothetical protein	4.7	1.4	
YPO3522		Conserved hypothetical protein	1.5	1.9	
YPO3534		Putative exported protein	1.9	2.2	
YPO4070	<i>viaF</i>	Putative exported protein	1.7	8.9	-3.8

*Pseudogene.

†YPO0118, YPO0756, YPO0997, YPO3491, YPO3703, YPO3932, YPO4047, YPO4092

genes specifically expressed in each growth phase, we compared the transcriptional profiles of *Y. pestis* harvested during the exponential and stationary phases in human plasma.

Genes showing transcriptional induction in human plasma during the exponential phase. Fifty-eight genes induced in human plasma were upregulated during the exponential phase (Table 3). The most represented functional category (20 genes) was that corresponding to unknown functions, followed by genes involved in iron metabolism (11 genes).

The outer-membrane lipoprotein-encoding gene *lpp* was the third most highly expressed gene during growth of *Y. pestis* in human plasma (Supplementary Table S4). A proteome analysis showed that Lpp is highly immunogenic upon *Y. pestis* EV76 injection into rabbits (Li *et al.*, 2005). Therefore, Lpp may represent a major constituent of the *Y. pestis* membrane, and may be important at various steps of the disease. In fact, virulence of *Salmonella enterica* is abolished by mutating one or the other of the two copies of the *lpp* gene (Sha *et al.*, 2004).

Genes showing transcriptional induction in human plasma during the stationary phase. Fifty-nine genes induced in human plasma were upregulated during the stationary phase (Table 4). Genes encoding proteins of unknown function were the most numerous (26). Of these, YPO1994 had been previously identified by a signature-tagged mutagenesis (STM) screen following subcutaneous infection of mice with *Y. pestis* Kimberley53 (Flashner *et al.*, 2004). The YPO1994 mutation did not affect mouse

mortality but decreased the capacity of the bacterium to colonize the spleen. YPO2976 (which encodes another hypothetical protein of unknown function) has been shown to be induced *in vivo* in the rat bubo (Sebbane *et al.*, 2006b) and in the mouse lung (Lathem *et al.*, 2005; Sebbane *et al.*, 2006b), suggesting that this gene is important in all stages of the infection process.

While several iron-uptake genes were induced during the exponential growth phase in human plasma (Table 3), three genes, *znuA*, *mntH* and YPO2332, involved in zinc, manganese and magnesium uptake, respectively, were specifically induced during the stationary phase (Table 4). Transcription of *znuA* has also been shown to be induced *in vivo* during bubonic and pneumonic murine plague (Lathem *et al.*, 2005; Sebbane *et al.*, 2006b), suggesting that this zinc-uptake system may be essential for *Y. pestis* at various stages of the disease. A role in virulence has been demonstrated for other pathogens, such as *S. enterica*, *Pasteurella multocida* and *Brucella abortus* (Campoy *et al.*, 2002; Garrido *et al.*, 2003; Kim *et al.*, 2004).

In contrast with the exponential-phase results, only two genes (*irp2* and *bfr*) involved in iron metabolism were induced during the stationary phase (Table 4). The *bfr* gene encodes bacterioferritin, which, in association with Bfd ferredoxin, is thought to release iron under iron-restriction conditions. Although the *bfd* gene was not significantly induced during the stationary phase, it was 3.2-fold induced in human plasma (Supplementary Table S1). This is in agreement with previous results showing that *bfr* and *bfd* are upregulated in *Y. pestis* biovar Microtus strain 201 during iron depletion (Zhou *et al.*, 2006).

Table 4. Genes exhibiting transcriptional induction in human plasma during the stationary phase

Ratios (*R*) were calculated by pairwise comparison of growth conditions: human plasma vs rich medium (HP:LB), mid-exponential phase vs stationary phase (expo:stat), and 37 vs 28 °C (37:28). Absence of a value indicates that the difference was not significant ($P \geq 0.05$).

Gene ID	Name	Function	<i>R</i> (HP:LB)	<i>R</i> (expo:stat)	<i>R</i> (37:28)
Iron metabolism					
YPO0206	<i>bfr</i>	Bacterioferritin	4.5	−2.4	
YPO1911	<i>irp2</i>	Yersiniabactin biosynthetic protein	7.1	−1.4	2.5
Non-iron metallic element metabolism					
YPO2061	<i>znuA</i>	Exported high-affinity zinc uptake system protein	2.2	−1.6	
YPO2332		Putative Mg ²⁺ transporter, MgtC family	1.9	−1.6	−1.4
YPO2982	<i>mntH</i>	Manganese transport protein MntH	3	−1.4	
Stress-responsive proteins					
YPO1134	<i>psiF</i>	Putative starvation-inducible protein	1.8	−1.7	
YPO1957	<i>phoH</i>	Putative phosphate starvation-inducible protein	2	−2.1	−1.6
YPO2229	<i>osmB</i>	Osmotically inducible lipoprotein B precursor	1.8	−1.8	
YPO2301		Putative stress protein	2.7	−2.3	
YPO2510	<i>dps</i>	Putative starvation-inducible DNA-binding protein	4	−2.4	
YPO3969	<i>uspB</i>	Universal stress protein B	2.5	−2.8	
Carbohydrate metabolism					
YPO1177	<i>dld</i>	D-Lactate dehydrogenase	2.3	−2.8	
YPO1358	<i>poxB</i>	Pyruvate dehydrogenase (cytochrome)	2.3	−1.9	
YPO3090	<i>ushA*</i>	UDP-sugar hydrolase	1.8	−1.5	
Metabolism of vitamins					
YPO1603	<i>pabC</i>	4-Amino-4-deoxychorismate lyase	1.8	−1.4	1.4
Outer-membrane or secreted proteins					
YPO0130		Putative secreted protein	1.9	−2	−2
YPO1005		Putative antigenic leucine-rich repeat protein	1.9	−1.6	
YPO1006		Putative antigenic leucine-rich repeat protein	1.3	−1.4	
YPO2202		Putative lipoprotein	2.1	−2.4	−1.8
Regulators					
YPO0883		Putative DNA-binding protein	1.7	−1.5	
YPO1648		Probable histidine acid phosphatase	1.9	−1.8	
YPO4028		Putative regulator	1.6	−1.5	
Miscellaneous					
YPO0198		Putative intracellular sulphur oxidation protein	2.6	−1.7	2.6
YPO0199		Putative intracellular sulphur oxidation protein	1.8	−1.4	1.7
YPO0676		Putative aldo/keto reductase family protein	3.1	−2.1	
YPO0700		Fimbrial protein	1.5	−1.5	
YPO1980		Zn-dependent hydrolase	1.6	−1.7	
YPO2151		Putative cobalt-nickel resistance (export) protein	1.7	−2	
YPO2153		Serine protein kinase	2.3	−2.1	
YPO3198	<i>ggt*</i>	Gamma-glutamyltranspeptidase precursor	2	−1.7	
YPO3583	<i>yhbH*</i>	Probable σ^{54} modulation protein	2.3	−1.4	−1.5
YPO3687	*	Putative aldehyde dehydrogenase	2	−1.6	
YPO3689		Putative RNase	1.6	−1.4	
Unknown					
YPMT1.71		Hypothetical protein	1.8	−2	
YPMT1.78	*	Hypothetical protein	1.8	−1.8	
YPO0194		SlyX protein	2.3	−2	
YPO0323		Conserved hypothetical protein	1.7	−1.6	
YPO0544		Putative inner membrane protein	1.5	−1.7	
YPO1180		Hypothetical protein	2.2	−1.8	
YPO1181		Putative membrane protein	4.3	−1.5	−1.9
YPO1832		Hypothetical protein	2.2	−1.7	−1.5
YPO1833		Hypothetical protein	1.7	−1.4	
YPO1956		Hypothetical protein	2.2	−1.7	−1.7
YPO1994		Hypothetical protein	2.7	−3.1	
YPO1995		Hypothetical protein	2.8	−2.9	

Table 4. cont.

Gene ID	Name	Function	R (HP:LB)	R (expo:stat)	R (37:28)
YPO1996		Hypothetical protein	2	-2.4	
YPO2092		Hypothetical phage protein	1.9	-2.5	
YPO2152		Conserved hypothetical protein	2	-2.4	
YPO2362		Putative membrane protein	1.9	-1.8	
YPO2363		Conserved hypothetical protein	1.4	-1.7	
YPO2435		Putative membrane protein	2	-1.7	
YPO2606		Conserved hypothetical protein	2.7	-1.6	2
YPO2611		Conserved hypothetical protein	1.6	-1.6	
YPO2820		Hypothetical protein	1.9	-2.5	
YPO2854		Conserved hypothetical protein	2	-1.9	-1.7
YPO2976		Conserved hypothetical protein	2.6	-3.1	
YPO2987		Putative exported protein	1.7	-1.6	
YPO3027	<i>ygiW</i>	Putative exported protein	3.7	-1.5	-1.8
YPO3527		Conserved hypothetical protein	3.6	-5.1	1.6

*Pseudogene.

Regulation of genes carried on the two *Y. pestis*-specific plasmids

Y. pestis classically harbours three plasmids: the virulence plasmid pCD1 (70.3 kb), which is common to the three pathogenic *Yersinia* species, and two *Y. pestis*-specific plasmids, pMT1 (96.2 kb) and pPCP1 (9.6 kb). In order to investigate the role of these plasmids during the septicaemic phase of plague, we analysed the transcriptional regulation of pMT1- and pPCP1-borne genes. None of the three pPCP1 genes (*pst*, *pim* and *pla*) spotted on macroarrays was significantly induced in human plasma. Of the 69 pMT1 genes spotted on the macroarrays, only two (YPMT1.71 and the pseudogene YPMT1.78) were induced in human plasma (both 1.8-fold). YPMT1.71 encodes a hypothetical protein which generates an antibody response after rabbit immunization with *Y. pestis* EV76 (Chen *et al.*, 2006). All *caf* genes (encoding the F1 capsular antigen) were induced at 37 °C. The upregulation values of *cafI* (41.5-fold) and *cafIA* (22.7-fold) were among the highest encountered in the whole genome. This strong temperature induction has also been observed in *Y. pestis* strains KIM5 D27 and 201, and agrees with the 37 °C-inducible antiphagocytic property of the F1 capsular antigen (Du *et al.*, 2002; Han *et al.*, 2004; Motin *et al.*, 2004). The *ymt* gene, encoding a phospholipase D which is not a virulence factor in mice but which is important for flea-borne transmission (Hinnebusch *et al.*, 2000, 2002), was constitutively expressed in human plasma and LB.

In conclusion, a transcriptome approach was used for the identification of genes induced during growth of *Y. pestis* in human plasma. Several of the identified genes potentially represent new virulence factors active in the septicaemic phase of plague in humans. They may be important once the bacteria have reached the bloodstream, by ensuring *Y. pestis* survival during infection (protection against oxidative and other stresses, cell wall accommodation), by

facilitating its proliferation (metabolic adaptation), or by damaging the host (adhesins, invasins and lytic enzymes such as proteases and phospholipases). Further characterization of these genes and their products may provide insight into the mechanisms responsible for the very high pathogenic potential of *Y. pestis*.

ACKNOWLEDGEMENTS

This work was supported in part by grant 0234021 from the 'Délégation Générale pour l'Armement' and grant PTR88 from the 'Programmes transversaux de recherche', Institut Pasteur.

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Edited by: T. Abee