Temperature and growth phase influence the outer-membrane proteome and the expression of a type VI secretion system in *Yersinia pestis*

Rembert Pieper,¹ Shih-Ting Huang,¹ Jeffrey M. Robinson,¹ David J. Clark,¹ Hamid Alami,¹ Prashanth P. Parmar,¹ Robert D. Perry,² Robert D. Fleischmann¹ and Scott N. Peterson¹

Correspondence

Rembert Pieper
rpieper@jcv.org

¹J. Craig Venter Institute, 9712 Medical Center Drive, Rockville, MD 20850, USA

²Department of Microbiology, Immunology and Molecular Genetics, University of Kentucky, Lexington, KY 40536, USA

Yersinia pestis cells were grown *in vitro* at 26 and 37 °C, the ambient temperatures of its flea vector and its mammalian hosts, respectively, and subjected to subcellular fractionation. Abundance changes at 26 vs 37 °C were observed for many outer-membrane (OM) proteins. The cell adhesion protein Ail (y1324) and three putative small β -barrel OM proteins (y1795, y2167 and y4083) were strongly increased at 37 °C. The Ail/Lom family protein y1682 (OmpX) was strongly increased at 26 °C. Several porins and TonB-dependent receptors, which control small molecule transport through the OM, were also altered in abundance in a temperature-dependent manner. These marked differences in the composition of the OM proteome are probably important for the adaptation of *Y. pestis* to its *in vivo* life stages. Thirteen proteins that appear to be part of an intact type VI secretion system (T6SS) were identified in membrane fractions of stationary-phase cells grown at 26 °C, but not at 37 °C. The corresponding genes are clustered in the *Y. pestis* KIM gene locus y3658–y3677. The proteins y3674 and y3675 were particularly abundant and co-fractionated in a M_r range indicative of participation in a multi-subunit complex. The soluble haemolysin-coregulated protein y3673 was even more abundant. Its release into the extracellular medium was triggered by treatment of *Y. pestis* cells with trypsin. Proteases and other stress-response-inducing factors may constitute environmental cues resulting in the activation of the T6SS in *Y. pestis*.

Received 10 July 2008

Revised 5 September 2008

Accepted 23 September 2008

INTRODUCTION

Yersinia pestis, a Gram-negative bacterium, is the causative agent of bubonic and pneumonic plague. The pathogenic lifestyle of this microbe involves two distinct life stages, one in the flea vector, the other in mammalian hosts, primarily rodents (Brubaker, 2002). *Y. pestis* and *Yersinia pseudotuberculosis*, a less virulent gastrointestinal pathogen in humans, diverged from a common ancestor about 20 000 years ago. The *Y. pestis* strains associated with high virulence have been divided into three classical biovars (antiqua, mediaevalis and orientalis) based on differences in their abilities to ferment glycerol and reduce nitrate (Anisimov *et al.*, 2004; Wren, 2003; Zhou *et al.*, 2004a, b).

Abbreviations: 2DGE, 2D gel electrophoresis; CBB, Coomassie brilliant blue G250; CCS, cell culture supernatant; hpH-MBR, high-pH-extracted membrane; hs-MBR, high-salt-extracted membrane; usb-MBR, urea/thiourea/amidosulphobetaine-14-extracted membrane; IM, inner membrane; IPG, immobilized pH gradient; OM, outer membrane; SEC, size-exclusion chromatography; T3SS, type III secretion system; T6SS, type VI secretion system; TMD, transmembrane domain.

Complete DNA sequence data exist for the genomes of each of these three biovars (Chain *et al.*, 2006; Deng *et al.*, 2002; Parkhill *et al.*, 2001; Song *et al.*, 2004). The gene organizations and complete DNA sequences of three *Y. pestis* virulence-associated plasmids have also been determined (Hu *et al.*, 1998; Lindler *et al.*, 1998). The pCD1 plasmid present in all other human-pathogenic *Yersinia* species encodes a suite of proteins required for a functional type III secretion system (T3SS) and host infection. A temperature increase from 26–30 to 37 °C and host cell contact or low Ca^{2+} concentration induce expression of these proteins (Cornelis, 2002). Most *Y. pestis* strains harbour two unique plasmids, pPCP1 and pMT1, not present in *Y. pseudotuberculosis*. These plasmids encode factors such as the plasminogen activator protease (Pla), required for mammalian pathogenesis (Sodeinde *et al.*, 1992), the *Yersinia* murine toxin (Ymt), required for colonization of the mid-gut of fleas (Hinnebusch *et al.*, 2000, 2002), and the F1 capsular antigen (Caf1) (Zavialov *et al.*, 2003). F1 antigen provides *in vitro* resistance to

phagocytosis but its role in mammalian virulence is unclear (Davis *et al.*, 1996). The genetically unstable chromosomal 102 kb *pgm* locus is also important for full virulence of *Y. pestis* in mammals and for transmission via blocked fleas (Fetherston *et al.*, 1992; Hinnebusch *et al.*, 1996; Perry & Fetherston, 1997, 2004). It encodes the yersiniabactin siderophore-dependent iron transport (Ybt) system and the haemin storage system (Hms)-dependent biofilm system. Biofilm formation allows colonization of the flea proventriculus, causing blockage which induces active feeding behaviour (Hinnebusch, 2004; Jarrett *et al.*, 2004; Perry *et al.*, 2004).

Transcriptional microarray and proteomic studies have been performed to examine temperature-dependent gene/protein abundance changes on the global level in *Y. pestis* strains (Chauvaux *et al.*, 2007; Chromy *et al.*, 2005; Han *et al.*, 2004; Hixson *et al.*, 2006; Motin *et al.*, 2004). The majority of virulence-associated factors were differentially expressed at 26–30 vs 37 °C. In two microarray gene expression analyses, strong thermoregulation of ypo0498–ypo0516 in CO92 and y3658–3677 in KIM1+ were reported (Han *et al.*, 2004; Motin *et al.*, 2004). Based on recent sequence alignment data, the gene clusters are hypothesized to encode a putative *Y. pestis* type VI secretion system (T6SS). Experiments disrupting putative T6SS genes and examining protein secretion via T6SS activities have demonstrated that T6SSs of *Pseudomonas aeruginosa* (Mougous *et al.*, 2006), *Vibrio cholerae* (Pukatzki *et al.*, 2006), *Edwardsiella tarda* (Zheng & Leung, 2007) and *Burkholderia mallei* (Schell *et al.*, 2007) are important for virulence in their respective hosts. In *V. cholerae* strains causing human infections, proteins encoded by the T6SS locus were termed Vas proteins (Pukatzki *et al.*, 2007). Three *V. cholerae* proteins, VgrG-1, VgrG-2 and VgrG-3, were proposed to form a phage tail spike-like protein complex that allows translocation of VgrG-1 into macrophages, where it cross-links actin. The haemolysin-coregulated protein (HCP) was shown to be secreted by *V. cholerae* prior to its association with and the discovery of the T6SS (Williams *et al.*, 1996). In characterizing the T6SS of *P. aeruginosa*, specific protein phosphorylation events involving the ATPase ClpV1 have been linked to the translocation of HCP to the cell surface (Mougous *et al.*, 2007). The core structures of T6SS protein pumps have not been characterized to date. Interestingly, a recent review on evolutionary relationships in T6SSs suggests gene loci for five different T6SSs in *Y. pestis* (Bingle *et al.*, 2008).

By contrast, the T3SS is well characterized in several *Yersinia* species. The membrane channel-forming core is termed the injectisome and is composed of Ysc subunits that form a hetero-multimeric protein complex (Cornelis, 2002). There is evidence for the involvement of adhesion proteins (YadA, Inv, Ail) in establishing contact between bacteria and host cells, before the translocator pore of the T3SS, in which LcrV has a critical function, assembles and releases effector proteins (Cornelis, 2002). In *Y. pestis*,

YadA and Inv are inactivated. But the *Y. pestis* outer-membrane (OM) protein y1324 (termed OmpX and Ail), was recently associated with adherence to and internalization of *Y. pestis* by cultured epithelial cells (Kolodziejek *et al.*, 2007). A different study reported that complement-dependent killing and human serum resistance of *Y. pestis* was mediated by Ail (Bartra *et al.*, 2008). Cell surface localization was also reported for the OM proteins Pla, Lpp, Ail and Pcp (Myers-Morales *et al.*, 2007).

Our objectives were to determine temperature-dependent protein abundance changes in membrane fractions of the *Y. pestis* strain KIM6+, and to examine these data in the context of the proteins' subcellular localizations, potential functional roles and participation in multi-subunit complexes. Changes in the OM proteome and expression of proteins that are part of a putative T6SS emerged as the most interesting features of this survey.

METHODS

Bacterial strains and culture conditions. The *Y. pestis* strain KIM6+ used in this study is an avirulent derivative of the fully virulent KIM strain, which was cured of the pCD1 plasmid but retained the chromosomal *pgm* locus and plasmids pMT1 and pPCP1 (Fetherston & Perry, 1994). We used strain maintenance and cell growth procedures and verified the presence of the *pgm* locus in Congo red agar as described previously (Lillard *et al.*, 1999; Pieper *et al.*, 2008). Briefly, bacterial colonies were grown on tryptose blood agar, harvested after 48 h and stored at –80 °C. Aliquots of these cell stocks were used to grow 5–10 ml pre-cultures in chemically defined medium (PMH2) for 8–15 h, followed by dilution into 0.5–1 l PMH2 containing 2.5 M K₂HPO₄ and 10 µM FeCl₃. Overnight cell cultures were grown to an OD₆₀₀ of ~1.9–2.5. Cells were also grown in P_i-limited PMH2 (0.12 M K₂HPO₄) to an OD₆₀₀ of ~1.0–1.4 and in iron-depleted PMH2 to an OD₆₀₀ of ~0.5–0.8 overnight. Iron impurities were removed from PMH2 by incubation with Chelex-100 resin for 20 h at 4 °C. Finally, cells were grown in brain heart infusion broth to an OD₆₀₀ of ~2.2. All cell cultures were performed at 26 and 37 °C. Cell pellets were harvested by centrifugation at 8000 g for 15 min at 4 °C and washed with about 30 volumes of 33 mM K₂HPO₄ (pH 7.5).

Subcellular fractionation of *Y. pestis* cells. Periplasmic fractions of *Y. pestis* KIM6+ cells were generated using a lysozyme/EDTA spheroplasting method, followed by lysis of spheroplasts via sonication in a hypotonic buffer as previously described (Lucier *et al.*, 1996; Pieper *et al.*, 2008). Soluble periplasmic and cytoplasmic fractions were exchanged into buffer A (25 mM NH₄HCO₃, pH 7.8, 1 mM Na₂EDTA and 1 mM benzamidine) and concentrated to 2–5 mg protein ml^{–1} at 3000 g using membrane filtration units (NMWL 10 000). Unless stated otherwise, proteins recovered from other subcellular and chromatographic fractions were concentrated accordingly. Mixed-membrane pellets were separated from soluble cytoplasmic fractions by centrifugation at 50 000 g for 1 h at 4 °C. Sucrose gradient centrifugation was used for a crude separation of OM and inner membrane (IM) fractions from cells grown at 26 °C. In this case, spheroplasts were lysed in a hypotonic buffer (25 mM Tris/acetate, pH 7.8, 5 mM Na₂EDTA and 0.2 mM DTT) using three freeze/thaw cycles over 2 h rather than sonication. Incubation of the lysate with 5 µg ml^{–1} each of DNase I and RNase in the presence of 10 mM MgCl₂ at 20 °C for 1 h was followed by centrifugation at 4000 g for 15 min at 4 °C. The membrane pellet was homogenized in

15% (w/v) sucrose and a discontinuous density-gradient centrifugation with 15, 53 and 70% sucrose layers was performed at 108 000 *g* for 17 h as previously described (Black *et al.*, 1987).

KIM6+ cells grown to stationary phase at 26 and 37 °C in PMH2 were also subjected to extraction of cell surface-associated proteins with 1 M NaCl, and the cell culture supernatants (CCSs) were collected. Detailed sample preparation methods were previously published (Pieper *et al.*, 2008). Finally, stationary-phase cells grown at 26 °C were digested with trypsin (100 µg per ml cell suspension) in Tris/acetate-buffered 0.25 M sucrose (pH 7.8). After gentle agitation for about 15 h at 37 °C, 10 mM Na₂EDTA was added. Supernatants were immediately separated from cell pellets by centrifugation at 8000 *g* for 15 min, filtered through a 0.45 µm PVDF membrane and concentrated to 1–2 mg protein ml⁻¹. Each of the two aforementioned fractions was also generated from cell cultures at 26 °C in brain heart infusion broth.

Membrane protein extraction. Mixed-membrane and OM pellets were resuspended and homogenized in 0.25 M sucrose, 150 mM NaCl, 10 mM Tris/acetate (pH 7.8), 5 mM EDTA, 0.2 mM DTT, 10 µg Leupeptin ml⁻¹, 5 µg Pepstatin ml⁻¹, 10 µg TAME (*N*^α-*p*-tosyl-L-arginine methyl ester) ml⁻¹ and 2 mM PMSF (~10 ml per g pellet). NaBr (2.5 M final concentration) was added; membrane homogenates were stirred for 1 h at 20 °C and centrifuged at 50 000 *g* for 1 h at 4 °C. Proteins in high-salt-extracted membrane supernatant (hs-MBR) fractions were concentrated to 1–2 mg ml⁻¹. Insoluble pellets were rehomogenized in ice-cold solutions of 0.18 M Na₂CO₃ (pH 11.3), 50 mM DTT, 1 mM CaCl₂, 1 mM MgCl₂ and 1 mM MnCl. The membrane suspensions were stirred for 1 h at 4 °C, followed by centrifugation at 50 000 *g* for 1 h at 4 °C. The supernatants, extracted at this high pH and termed hpH-MBR fractions, were concentrated to ~1–2 mg protein ml⁻¹. Prior to sample analysis in 2DGE gels, insoluble membrane pellets were solubilized with 8 M urea, 2 M thiourea, 1% (w/v) amidosulfobetaine-14, 2 mM tributylphosphine and 0.5% Bio-Lyte pH 3–10 carrier ampholytes. Following incubation for 30 min at 20 °C and centrifugation at 16 200 *g* for 15 min, remaining pellets were discarded. Aliquots of supernatants, termed the usb-MBR fractions, were run in SDS-PAGE gels to approximate protein concentrations.

Size-exclusion chromatography (SEC) experiments. The hpH-MBR fraction derived from cells cultured at 26 °C was concentrated to ~1 mg ml⁻¹ and Triton X-100 (0.075% w/v) was added. An aliquot of ~1 ml was applied to a Superdex 200 column (1.6 × 100 cm) equilibrated in 100 mM Na₂HPO₄ (pH 7.5), 150 mM NaCl, 2 mM TAME, 2 mM Na₂EDTA and 0.05% Triton X-100. The flow rate was set at 0.8 ml min⁻¹ and proteins were fractionated by FPLC at 20 °C. To calibrate the column (elution time vs *M_r* value of all proteins), a standard mixture of thyroglobulin (670 kDa), bovine IgG (158 kDa), ovalbumin (45 kDa), myoglobin (17 kDa) and vitamin B12 (1.4 kDa) was applied. Protein elution was monitored as A₂₈₀. Fractions of 6 ml were collected and concentrated to ~1–2 mg ml⁻¹. Separate SEC experiments were carried out to determine oligomeric states of the extracellular T6SS subunit HCP. Aliquots (~75 µl) of three KIM6+ subcellular fractions concentrated to 5 mg ml⁻¹ were applied to a 4.6 mm × 30 cm TSKgel SW3000 column: (1) the periplasmic supernatant; (2) the supernatant of KIM6+ cells incubated with trypsin for 15 h; (3) the periplasmic fraction of the aforementioned trypsin-treated cells. At a flow rate of 0.8 ml min⁻¹, proteins were eluted and analysed by SDS-PAGE and 2DGE. The aforementioned protein standards were used for the *M_r* calibration of the SW3000 column.

Protein separation and differential protein spot display in 2DGE gels. Samples of ~75 µg protein for Sypro Ruby-stained gels and ~130 µg for Coomassie brilliant blue G250 (CBB)-stained gels

were loaded onto 24 cm IPG gel strips (pH range 4–7 or pH range 3–10 IPG strips) and separated in the first dimension as described previously (Pieper *et al.*, 2008). Second-dimension slab gels (25 × 19.5 × 0.15 cm), gel staining/imaging and differential spot display with the gel image analysis software Proteomweaver vs4.0 were also performed as described (Pieper *et al.*, 2008). Six distinct *Y. pestis* KIM6+ growth conditions resulted in six usb-MBR fractions and six different groups of gel images. Each group was represented by at least three pH range 4–7 gels: (1) growth at 26 °C in complete PMH2; (2) growth at 37 °C in complete PMH2; (3) growth at 26 °C in P_i-limited PMH2; (4) growth at 37 °C in P_i-limited PMH2; (5) growth at 26 °C in iron-depleted PMH2; (6) growth at 37 °C in iron-depleted PMH2. In binary differential spot display experiments, protein abundance ratios were measured to determine temperature-dependent changes. The analyses were group 1 vs 2, group 3 vs 4 and group 5 vs 6. For spot normalization in gels, an adjustment factor was included based on the measurement of total spot intensities in gel images for the 4–7 vs the 7–10 pH range. In addition, differential display was performed for one set of usb-MBR fractions in the pH range 7–10 and one set of hs-MBR fractions in the pH range 4–7 (group 1 vs 2 in each case). Criteria for statistical significance of spot abundance changes in non-parametric *t*-tests were set at a *P*-value of <0.01 and an abundance ratio >1.5.

Mass spectrometry (MS) and bioinformatic protein analysis.

Methods for spot cutting and protein digestion with trypsin were reported previously (Pieper *et al.*, 2008). Peptide digests were analysed using a MALDI-TOF/TOF mass spectrometer (4700 Proteomics Analyser, Applied Biosystems) and a nano-electrospray LC-MS/MS system (LTQ ion trap mass spectrometer, Thermo-Finnigan) equipped with an Agilent 1100 series solvent delivery system. Reversed-phase peptide separations for LC-MS/MS analysis were performed at nanoflow rates (350 nl min⁻¹). Further technical details of mass spectrometry (MS) and tandem MS analyses were described previously (Gatlin *et al.*, 2006). Data were searched against the latest release of the *Y. pestis* KIM strain subset of the NCBI nr database, using the Mascot search engine vs2.1 (Matrix Science). Carbamidomethyl was invariably selected as a fixed modification and one missed tryptic cleavage was allowed. MALDI search parameters (+1 ions) included mass error tolerances of ±100 p.p.m. for peptide ions and ±0.2 Da for fragment ions. LTQ ion-trap search parameters (+1, +2 and +3 ions) included mass error tolerances of ±1.4 Da for peptide ions and ±0.5 Da for fragment ions. Protein identifications were accepted as significant when a Mascot protein score >75 and at least one peptide *e*-value <0.1 per 2DGE spot were obtained. Using a randomized decoy database and a default significance threshold of 0.05 in Mascot, the false-positive rate for peptides identified by LC-MS/MS was ~6%. Since we identified at least two peptides per 2DGE spot in more than 95% of all cases, the true false positive rate was less than 0.3% for protein identifications. For bioinformatic predictions of lipoproteins, transmembrane domains (TMDs), export signal and β-barrel OM protein motifs, the following algorithms were used: LipoP, TMHMM, SignalP (<http://www.cbs.dtu.dk/services>) and PRED-TMBB (Bagos *et al.*, 2004), respectively.

RESULTS

Experimental approaches to profiling thermoregulated *Y. pestis* membrane proteins

Several characterized virulence factors expressed by the plague bacterium are known to be strongly thermoregulated, including the T3SS. Given the extensive investigation

of the T3SS, we used the strain *Y. pestis* KIM6+, which is cured of the T3SS-encoding pCD1 plasmid. This strain retains the important *pgm* pathogenicity island and the virulence-associated plasmids pMT1 and pPCP1. Recently, we globally profiled proteins residing in periplasmic and cell culture supernatant (CCS) fractions (Pieper *et al.*, 2008), and cytoplasmic and membrane-associated fractions (unpublished data) by 2DGE and MS analysis. Here, our first objective was to identify membrane-associated, thermoregulated proteins. Our second objective was to examine a set of strongly thermoregulated proteins whose corresponding genes were clustered. Conserved domains in most of the proteins established a link to a putative T6SS. Membrane pellets derived from *Y. pestis* cell lysates were sequentially extracted with high salt (2.5 M NaBr), at high pH (Na₂CO₃, pH 11.3) and with membrane denaturants (urea/thiourea, 1 % amidosulfobetaine-14). The fractions were named hs-MBR, hpH-MBR and usb-MBR, respectively, for further reference in this study. To examine subcellular localizations of an orthologue of HCP, the protein that is thought to be secreted by the T6SS in other bacteria, additional subcellular fractions were analysed by 2DGE and MS: the cytoplasm, the periplasm, the CCS and a trypsin-digested supernatant fraction.

For differential protein display at 26 vs 37 °C, we focused on the usb-MBR fraction. The fraction was enriched in integral OM proteins. Integral IM proteins, lipoproteins and peripheral membrane proteins were also identified. But IM proteins harbouring more than one predicted TMD were invariably excluded from quantitative analysis, since they lacked sufficient resolution and/or abundance in gels. The comparisons at 26 vs 37 °C pertained to *Y. pestis* cells grown overnight in P_i-limited medium, in iron-depleted medium or in chemically defined complete medium. To decrease experimental variability, the usb-MBR fractions were recovered from two or three separate cell culture batches for each of the six growth conditions. Equivalent fractions were pooled prior to protein separation in 2DGE gels. Stringent criteria for statistical significance (*t*-test *P*-values <0.01) were applied to determine differentially abundant spots. Phosphate limitation results in the reduction of intracellular ATP and GTP, which causes energy starvation. Iron depletion leads to oxidative stress in cells, because many oxidoreductive enzymes require iron as a cofactor. Such growth conditions are potentially useful to mimic nutrient-limited environments that *Y. pestis* encounters *in vivo*. Our rationale was that proteins altered in abundance at 37 vs 26 °C independent of other growth conditions are fundamentally important for one of the two life stages under consideration (mammal vs flea).

Thermoregulated membrane proteins with low variability in spot ratios comparing three growth conditions

Most proteins appeared as spot trains in 2DGE gels of usb-MBR and hpH-MBR fractions. MS data linked the spots

from a given train to deamidation events in amino acid side chains, which resulted from sample preparation procedures. The spots were not true post-translational protein variants. While it was not biologically meaningful to discriminate between spot ratios for the same spot train, they are denoted in Table 1 if they were statistically significant (e.g. for Pla, OmpA and y3609).

Strong protein abundance increases at 37 vs 26 °C for at least two of three growth conditions were determined for characterized virulence factors (Ail/y1324, #15; Pla, #55; spot numbers in this and the next section reference the numbering in Fig. 1 and Table 1), *Y. pestis* antigens in mammalian hosts (KatY, #12; Caf1, #54), proteins previously linked to increased abundance at 37 °C compared to lower temperatures in Gram-negative bacteria (DegQ, #4; GroEL, #8; OmpC, #36) and proteins for which a context between temperature regulation and function has not been elucidated to date (NlpB, #17; y1795, #21; y2167, #25; y4083, #52). Strong protein abundance increases at 26 vs 37 °C for at least two of three growth conditions were determined for Ymt, a protein responsible for survival of *Y. pestis* in the flea (#13), Hms proteins known to be implicated in biofilm formation in the flea (HmsF, #26; HmsH, #27), an OM porin known to be induced at lower growth temperatures in Gram-negative bacteria (OmpF, #34) and proteins for which a connection between temperature regulation and function has not been elucidated to date (PldA, #7; GuaB, #16; OmpX/y1682, #20; y2104, #24). Also of note, four subunits of a putative *Y. pestis* T6SS (ClpB2/y3669, #44; y3673, #45; y3674, #46; y3675, #47) were detected at 26 °C, but not at 37 °C.

Since several peripheral membrane proteins differed in abundance at 26 vs 37 °C in the usb-MBR fraction, we were also interested in assessing such protein changes in the hs-MBR fraction. In most instances, only moderate variations were observed for proteins profiled in the equivalent hs-MBR and usb-MBR fractions. When comparing 21 proteins differentially expressed at 26 vs 37 °C for the two fractions (same growth condition, two rightmost columns in Table 1), quantitative differences were observed, but these were invariably less than 2.5-fold. The exceptions were the cytoplasmic enzyme Fba (#39) and a putative *N*-acetylmuramoyl-L-alanine amidase (y1845, #22) which may be involved in peptidoglycan degradation. With respect to the two life stages of *Y. pestis*, the changes in the abundance of small β -barrel OM proteins are most interesting. Ail was recently linked to processes contributing to the pathogenesis of *Y. pestis* in the mammalian host. The protein adheres to epithelial cells and initiates internalization. It is also responsible for resistance to killing by the human plasma complement system. Our data revealed that Ail is extremely abundant at 37 °C *in vitro* (~20–30 % of the total OM proteome) and much less abundant at 26 °C. In contrast, the Ail/Lom family protein OmpX showed the reverse abundance profile (~2–5 % of the total OM proteome at 26 °C).

Table 1. Quantitative changes in membrane-associated *Y. pestis* proteins isolated from three distinct growth states at 26 vs 37 °C

Spot no.*	Accession no. gi	Gene locus†	Gene name‡	Subcell. cat.§	Protein name and description	26 vs 37 °C low P _i	26 vs 37 °C low Fe¶	26 vs 37 °C usb-MBR#	26 vs 37 °C hs-MBR**
1	22123952	y0031	<i>malK</i>	IM	ATP-binding protein of maltose/maltodextrin ABC transporter			−1.7	
2	22123953	y0032	<i>lamB</i>	OM	Malto porin	<−10	−3.1		
3	22124056	y0136	(<i>yhcB</i>)	IM	Cytochrome <i>d</i> ubiquinol oxidase subunit III	2.3	1.9	1.5	
4	22124057	y0137	<i>degQ</i>	pM	Serine endoprotease	2.3		4	2.5
5	22124081	y0161	<i>pyrB</i>	pM	Aspartate carbamoyltransferase catalytic subunit	6.7		4.7	2.9
6	22124122	y0202	<i>mreB</i>	pM	Regulator of FtsI/penicillin-binding protein 3	3.1			
7	22124311	y0396	<i>pldA</i>	OM	OM phospholipase A	−4	−3.4	−6.3	
8	22124523	y0609	<i>groEL</i>	MSL	Chaperonin GroEL	6.7		7	3.1
9	22124636	y0722	<i>phnM</i>	pM	Phosphonate metabolism protein	−5			
10	22124731	y0818	<i>cysJ</i>	pM	NADPH-dependent sulfite reductase, flavoprotein β -subunit		<−10	<−10	−5
11	22124761	y0850	(<i>cirA2</i>)	OM	Putative TonB-dependent OM receptor for iron transport		3.2	−6.3	
12	22124781	y0870	<i>katY</i>	MSL	Catalase [hydroperoxidase HPI(I)]	2.6		2.6	6.3
13	31795399	Y1069	<i>ymt</i>	Cy	Yersinia murine toxin	−2.2	−4.8	−3.8	−1.7
14	22125123	y1221	<i>proV</i>	IM	ATP-binding subunit of glycine/betaine/proline transporter	−1.7		−2.3	−1.9
15	22125223	y1324	<i>ail</i>	OM	Attachment invasion locus protein Ail	6.7††	>1.5, n.s.v.	6.9††	
16	22125261	y1362	<i>guaB</i>	pM	Inositol-5-monophosphate dehydrogenase	−2.6		−7.8	−3.3
17	22125317	y1419	<i>nlpB</i>	OM	Lipoprotein NlpB	>1.5, n.s.v.		>1.5, n.s.v.	
18	22125472	y1577	<i>fadL</i>	OM	Long-chain fatty acid OM transporter			3.8	
19	22125474	y1579	<i>fadI</i>	pM	3-Ketoacyl-CoA thiolase	3.8		5.1	
20	22125577	y1682	<i>ompX</i>	OM	OM protein X	−7.7	−9.1	−4.9	−2.4
21	22125689	y1795	–	OM	Putative OM lipoprotein y1795	>10		>10	
22	22125738	y1845	–	pM	Probable <i>N</i> -acetylmuramoyl-L-alanine amidase; regulator			−3.5	2.6
23	22125812	y1919	(<i>wcaG9</i>)	M	Bifunctional UDP-glucuronic acid decarboxylase/formyltransferase		−2.3		
24	22125993	y2104	(<i>ydgA</i>)	pM	Putative phospholipid-binding lipoprotein y2104	−1.6		−2.1	
25	22126055	y2167	(<i>ompV</i>)	OM	Hypothetical protein; putative OM protein V	1.9		1.8, 6.4‡‡	
26	22126242	y2358	<i>hmsF</i>	OM	Haemin storage/biofilm formation protein HmsF	−2.2	−2	−2.7	
27	22126243	y2359	<i>hmsH</i>	OM	Haemin storage/biofilm formation protein HmsH	−8.1	−4.2	−5.2, <−10§§	
28	22126278	y2394	<i>ybtS</i>	pM	Salicylate synthase Irp9 (yersiniabactin biosynthesis)		−1.5		
29	22126286	y2402	<i>ybtT</i>	pM	Yersiniabactin thioesterase		−1.5		
30	22126287	y2403	<i>ybtE</i>	pM	Salicyl-AMP ligase (yersiniabactin biosynthesis)		−2.1		
31	22126288	y2404	<i>psn</i>	OM	Pesticin/yersiniabactin OM receptor	−4			
32	22126437	y2556	<i>fcuA</i>	OM	TonB-dependent OM ferrichrome receptor		2.1	4.8	
33	22126613	y2735	<i>ompA</i>	OM	OM protein A		−1.6, −3, −3.3‡‡		
34	22126637	y2759	<i>ompF</i>	OM	OM porin OmpF	<−10	−4.3	<−10	−14.3

Table 1. cont.

Spot no.*	Accession no. gi	Gene locus†	Gene name‡	Subcell. cat.§	Protein name and description	26 vs 37 °C low P _i	26 vs 37 °C low Fe¶	26 vs 37 °C usb-MBR#	26 vs 37 °C hs-MBR**
35	22126686	y2809	<i>clpA</i>	pM	ATP-binding subunit of serine protease	3.0			
36	22126843	y2966	<i>ompC</i>	OM	OM porin OmpC	3.1	3.4	2.6	7.5
37	22126860	y2983	<i>phoE</i>	OM	OM phosphoporin	-4.8			
38	22126930	y3054	<i>pal</i>	OM	Peptidoglycan-associated OM lipoprotein	<-10, n.s.v.		-6.3, n.s.v.	
39	22127183	y3307	<i>fba</i>	Cy	Fructose-bisphosphate aldolase	4.9	-2.7	6.4	1.5
40	22127186	y3310	<i>tktA</i>	Cy	Transketolase	6.5		5.1	
41	22127280	y3404	-	OM	Putative TonB-dependent OM receptor			>-10	
42	22127483	y3609	-	IM	Putative IM protein y3609	-5.3, -2.3‡‡			
43	22127491	y3617	<i>secA</i>	pM	Preprotein translocase subunit SecA	4	-3		-2.2
44	22127543	y3669	<i>clpB2</i>	pM	ATP-dependent protease (T6SS)	<-10	<-10	<-10	
45	22127547	y3673	-	MSL	Putative haemolysin-coregulated protein (T6SS)	<-10	<-10	<-10	-5.9
46	22127548	y3674	-	M	Putative T6SS subunit y3674	<-10, <-10§§	<-10	<-10, <-10§§	-6.3
47	22127549	y3675	-	pM	Putative T6SS subunit y3675	<-10		<-10	<-10
48	22127730	y3859	<i>uspA</i>	pM	Universal stress protein			1.7	1.5
49	22127786	y3916	<i>ompR</i>	pM	Osmolarity response regulator OmpR	4.8	2.7		1.5
50	22127855	y3985	<i>fusA</i>	MSL	GTP-binding protein chain-elongation factor (EF-G)	2, >10§§		4.8, >10§§	
51	22127856	y3986	<i>tufB</i>	MSL	Elongation factor Tu (EF-Tu)	3.2		4.5, >10§§	
52	22127952	y4083	-	OM	Putative secreted protein y4083	4.8	>10	4.6	
53	22127996	y4128	<i>pstB</i>	IM	ATP-binding protein of high-affinity phosphate ABC transporter	-2.1			
54	31795234	YPKMT065	<i>cafI</i>	OM	F1 capsule antigen	>10		>10	13.9
55	31795332	YPKp07	<i>pla</i>	OM	Plasminogen activator protease	4, 3.5‡‡		1.7, 2, 3.4‡‡	2

*Equivalent spot numbers are denoted in Fig. 1.

†Protein accession numbers and locus tags are from the KIM genome database (NCBI).

‡Names correspond to *Y. pestis* KIM locus tags; gene names in parentheses are from *E. coli* K-12, when >70 % sequence identity in at least 75 % of protein sequence.

§Subcellular category determined from experimental subcellular fractionation data and differential 2-DE display: Cy, cytoplasmic contaminant; IM, inner membrane; MSL, multiple subcellular localizations; M, membrane; OM, outer membrane; pM, membrane periphery (IM or OM).

||, ¶, #, **usb-MBR, urea/amidosulfofetaine-extracted membrane fraction; hs-MBR, high-salt-extracted membrane fraction; low P_i, P_i-limited PMH2 medium; low Fe, iron-depleted PMH2 medium; *n*-fold protein spot abundance differences with a *t*-test *P*-value <0.01; n.s.v., not statistically verified with a *P*-value <0.01, based either on irregular spot display or lack of resolution.

||Urea/amidosulfofetaine-extracted membrane fraction, 37 °C vs 26 °C (negative value when increased at 26 °C); cells were grown in PMH2 with 0.12 mM phosphate.

¶Urea/amidosulfofetaine-extracted membrane fraction, 37 °C vs 26 °C (negative value when increased at 26 °C); cells were grown in iron-depleted PMH2.

#Urea/amidosulfofetaine-extracted membrane fraction, 37 °C vs 26 °C (negative value when increased at 26 °C); cells were grown in complete PMH2.

**High-salt-extracted membrane fraction, 37 °C vs 26 °C (negative value when increased at 26 °C); cells were grown in complete PMH2 medium.

‡‡Spot abundance change was confirmed in 2-DE gels in pI range 6.5–10.

‡‡Spot abundance change for 1–3 spots in spot trains (from most acidic to most basic spot).

§§Spot change for protein fragment.

Fig. 1. Comparative analysis of membrane protein profiles derived from *Y. pestis* KIM6+ cells grown at 26 vs 37 °C. Three variations in growth conditions (overnight cultures) were examined: usb-MBR, -P_i, low-phosphate PMH2 medium (0.12 M K₂HPO₄); usb-MBR, -Fe, PMH2 medium depleted of iron (FeCl₃); usb-MBR, complete PMH2 medium. Proteins were separated in the pH range 4–7 and the *M_r* range 8–200 kDa in 2DGE gels. The gel images are representative of a set of three to five gels per group. Gels for usb-MBR, -Fe fractions were stained with Sypro Ruby. Other gels were stained with CBB. Quantitative data for spots denoted in the gel images are provided in Table 1 with equivalent spot numbers.

Two predicted Ail/Lom family proteins (y2034, y2446) were not detected in *Y. pestis* cells at either 26 °C or 37 °C. A conserved domain (MipA) suggests that the β -barrel protein y2167 is involved in cell envelope scaffolding. Two proteins with low M_r values, y1795 and y4083, have no sequence similarities to any other characterized proteins. *In silico* predictions by PRED-TMBB support the notion that y1795 and y4083 are small β -barrel OM proteins. In summary, we identified four small thermoregulated OM proteins with no known functions that, like Ail, may be cell-surface-exposed in *Y. pestis* during one of the two life stages.

Thermoregulated membrane proteins with higher variability in spot ratios comparing three growth conditions

For ~40 % of the 55 membrane-associated proteins with significant abundance changes at 26 vs 37 °C, spot ratios comparing the three growth conditions varied substantially. Fourteen proteins were annotated as putative regulators or were functionally linked to the acquisition and metabolism of nutrients (Table 1). Expression of such proteins is influenced by growth conditions associated with nutrient starvation. Cells grown to stationary phase or in P_i -limited medium revealed increased abundance of proteins involved in carbohydrate and/or phosphate uptake at 26 vs 37 °C. This included PstB, PhoE and PhnM, all of which are part of the Pho regulon and known to be induced in P_i -starved *Escherichia coli* cells, and MalK and LamB, both of which facilitate maltose import. In contrast, proteins involved in fatty acid import (FadL) and metabolism (FadI) were increased at 37 vs 26 °C in stationary-phase cells.

TonB-dependent OM receptors also displayed variability in abundance at 26 vs 37 °C. The only characterized TonB-dependent receptor was Psn (#31, Fig. 1), which is responsible for the uptake of the iron/yersiniabactin complex at the cell surface of *Y. pestis*. Psn was moderately increased at 26 vs 37 °C in P_i -starved cells. The putative OM receptor y0850 (#11) showed an unusual pattern of thermoregulation. This protein displayed sixfold higher abundance at 26 vs 37 °C in the OM of stationary-phase cells, threefold higher abundance at 37 vs 26 °C in the OM of iron-depleted cells and low expression in P_i -starved cells at both temperatures. While high expression of y0850 in iron-starved cells suggested a potential iron-uptake activity, adjacent genes did not reveal conserved motifs typical for an operon involved in metal ion transport. Expression of two putative OM receptor proteins, the ferrichrome receptor FcuA (#32) and y3404 (#41), was also strongly influenced by temperature and growth conditions. We hypothesize that abundance differences of TonB-dependent receptors in the OM alter the means by which *Y. pestis* imports metal ion complexes in vector versus host environments.

Membrane localization of putative *Y. pestis* T6SS proteins

The *Y. pestis* KIM gene locus y3658–y3677 encodes 18–20 proteins, of which at least 11 have been tentatively linked to a T6SS, based on COG domains conserved among multiple organisms. Thirteen proteins (gene products) were identified in this study and, with the exception of the proteins y3673 (also termed HCP here) and y3663, were enriched in membrane fractions (Table 2). Expression of the 13 corresponding genes was strongly repressed in cells grown at 37 °C. For reference purposes, gene locus tags of orthologous proteins in *P. aeruginosa* PAO1 are also listed in Table 2. Two *P. aeruginosa* proteins, HCP (PA0085) and VgrG-2 (PA0095), are thought to represent proteins secreted by the T6SS and to have effector functions in host cells (Mougous *et al.*, 2006). The *Y. pestis* VgrG-2-like protein y3668 was localized in the usb-MBR fraction, indicating strong membrane association. In contrast, HCP was a soluble protein.

All of the following T6SS characterization experiments were repeated at least twice. As shown in Fig. 2, many putative T6SS subunits were identified from gel spots of the hpH-MBR fraction. Alkaline extraction of membranes primarily results in the solubilization of peripheral and monotopic IM proteins (Molloy, 2008; R. Pieper, unpublished data). However, two low-abundance T6SS proteins with TMD motifs (y3658 and y3659) were also detected and displayed M_r and pI values indicative of proteolytic cleavage. These proteins are similar to VasK and VasF of *V. cholerae*, respectively, and essential for a fully functional T6SS in this pathogen. Three proteins (y3674, y3674F and y3675) were among the most abundant proteins in the hpH-MBR fraction. Their enrichment in this compared to the usb-MBR and hs-MBR fractions suggested monotopic-integral membrane association (gel montage, Fig. 3). ProV, the ATP-binding subunit of an amino acid ABC transporter, is a monotopic IM protein and showed a spot intensity distribution comparable to that of y3674 and y3675. In contrast, the integral OM protein OmpA and the peripheral IM proteins AtpA and ManX displayed different spot intensity distributions (Fig. 3A–C).

To assess whether some of the T6SS proteins also partitioned into OM fractions, a sucrose density gradient layer banding at a density characteristic for OMs (1.25 g ml⁻¹) was isolated. The fraction was indeed enriched in OM proteins, but still showed minor contamination with IM-associated proteins. Measuring spot quantities of 33 abundant integral OM proteins and 33 potential contaminant spots from the cytoplasm and the IM, the total OM spot quantity amounted to 91 % (mean CV of 47 %, $n=3$). Spots of y3674 and y3675 were decreased in this compared to the mixed-membrane usb-MBR fraction, as were AtpA, ManX and ProV (Fig. 3C, D). These data and the detection of tryptic peptides close to the N terminus by MS (Table 2) are in support of the localization of y3674 and y3675 in the *Y. pestis* IM. Most proteins translocated to the OM via Sec

Table 2. Subunits of the *Y. pestis* KIM T6SS

Abbreviations: F, protein observed as a fragment in 2DGE gels; TOF, peptide detected from MALDI-TOF data.

Accession no. gi	Locus tag*	Locus tag-PA†	T6SS	Subcell. cat.‡	Protein description (sequence similarity to orthologues and experimental data)§	COG	Obs. pI 2DGE	Obs. M _r 2DGE	Predict. pI¶	Predict. M _r ¶	N-terminal peptide#	Mascot score**
22127532	y3658	PA0077	y	IM	VasK/IcmF-like integral membrane protein	3523	5.9 _F	33 _F	9	133	N ₁₇₄ -R ₁₈₄	85
22127533	y3659	PA0078	y	IM	VasF-like integral membrane protein	3455	6.7 _F	46 _F	8.6	62	L ₇₂₁ -K ₇₃₄	27
22127534	y3660	PA0079	?	M	Integral membrane protein	—	5.3	45	5.6	51	M ₁ -K ₁₀	0 _{TOF}
22127537	y3663	—	?	Ex	Putative secreted protein	—	6.3	23	8	22	T ₂₄ -K ₃₉	20
22127538	y3664	—	?	M	Integral membrane protein with pentapeptide repeats	1357	5.7	43	5.7	42	M ₁ -R ₁₅	61
22127539	y3665	—	?	M	Integral membrane protein with pentapeptide repeats	1357	5.5	86	5.4	85	L ₂₃₁ -R ₂₅₁	20
22127540	y3668	PA0095	y	M	VgrG-like integral membrane protein	3501	5.2	98	5.2	86	V ₅₆ -R ₆₇	27
22127543	y3669	PA0090	y	pIM	ATP-dependent protease ClpB2	542	5.7	97	5.6	97	T ₂₃ -R ₂₉	46
22127545	y3671	PA0088	y	M	VasA-like integral membrane protein	3519	6.1	69	6.3	69	V ₁₁₇ -R ₁₂₇	69
22127547	y3673	PA0085	y	C, P, Ex	Secreted haemolysin-coregulated protein HCP	3157	5.2	18	5.2	19	W ₄₂ -K ₅₂	50
22127548	y3674	PA0084	y	mIM	Putative monotopic IM protein	3517	5.3	60	5.3	57	A ₇ -K ₂₉	92
22127548	y3674	PA0084	y	mIM	Post-translational variant of y3674	3517	5.6 _F	54 _F			R ₄₆ -K ₅₆	69
22127549	y3675	PA0083	y	mIM	Putative monotopic IM protein	3516	4.6	23	4.5	21	V ₁₉ -K ₃₂	90
22127550	y3676	PA0082	y	M	ImpA-like integral membrane protein	3515	4.7	41	4.8	39	L ₆₅ -R ₇₃	62

*Locus tag in *Y. pestis* KIM genome.†Locus tag for T6SS orthologues in *P. aeruginosa* PAO1 genome.

‡Proposed subcellular localization: IM, inner membrane; M, inner or outer membrane; C, cytoplasm; Ex, extracellular; P, periplasm; pIM, peripheral IM; mIM, monotopic IM.

§Orthologues from *P. aeruginosa* or *V. cholerae*.

||COG domain numbers.

¶M_r and pI values predicted from gi| annotations.

#Most N-terminal peptides identified by LC-MS/MS.

**MS/MS score for N-terminal peptides (Mascot data).

Fig. 2. Membrane-associated protein subunits of a putative T6SS in *Y. pestis* profiled in the hpH-MBR fraction. The fraction was derived from crude membranes of cells grown at 26 °C and extracted with 0.18 M Na₂CO₃ (pH 11.3). The 2DGE gel run conditions are described in the text. The gel was stained with CBB. Proteins are denoted with *Y. pestis* KIM locus tags. (F), protein was identified as a fragment.

or Tat secretion pathways have signal peptides of ~25–50 amino acids, which are cleaved from their N termini. In fact, none of the 13 putative T6SS proteins was predicted to have signal peptides. A fragment of y3674 (y3674F, Fig. 2) was shown to be cleaved at its N terminus. MS data placed the most N-terminal tryptic peptide of y3674F at R₄₆–K₅₆. A loss of ~40 amino acids from the N terminus was in agreement with its 2DGE spot position. There was no evidence suggesting translocation of the N-terminally processed y3674F to the OM.

Is the *Y. pestis* T6SS structurally and functionally intact?

The assigned functional role for y3669 was that of an ATP-dependent protease (ClpB2). ClpB2 was less abundant than y3674 and y3675 in membrane fractions and was also

detected in the cytoplasm, suggesting peripheral association with the IM in *Y. pestis*. The enzyme has extensive sequence similarity with ATPase subunits of ClpA/ClpB-type proteases and ClpV1, a T6SS-associated ATPase expressed by *P. aeruginosa*. In analogy to ClpV1, ClpB2 may form a cytoplasmic subunit that associates with integral IM subunits of the T6SS and produces energy for protein translocation processes (Mougous *et al.*, 2007). To examine the existence of a high-*M_r* multi-subunit T6SS complex, the hpH-MBR fraction was further fractionated by SEC. The proteins y3674, y3674F, y3675 and ClpB2 co-fractionated in the *M_r* range between ~500 and 200 kDa (data not illustrated here). For ClpB2, a 97 kDa protein thought to be homo-oligomeric, these data did not demonstrate its participation in a larger complex. While these data did not reveal direct interactions between y3674 and y3675, these proteins appear to be part of a

Fig. 3. Spot intensity distribution of the protein y3674 in four subcellular fractions. A montage view of CBB-stained gel segments in the 35–60 kDa range is provided: (A) hs-MBR (high-salt membrane extraction); (B) hpH-MBR (alkaline Na₂CO₃ membrane extraction); (C) usb-MBR; and (D) usb-MBR_OM (urea/amidosulfobetaine-14 membrane extractions). The fraction in gel (D) was derived from an OM-enriched sucrose density-gradient centrifugation band. The protein spots denoted in the gels are y3674, y3674F (a fragment of y3674), the peripheral membrane proteins AtpA and ManX, the monotopic membrane protein ProV (an ABC transporter subunit) and the integral OM protein OmpA.

multi-subunit complex. Of note, subunits of known IM protein complexes, e.g. ATP synthase, pyruvate dehydrogenase and tetrameric ATP transporters, were detected in the same M_r range.

HCP has been described as a secreted/extracellular protein in the context of the T6SSs of *P. aeruginosa* (Mougous *et al.*, 2007), *V. cholerae* (Williams *et al.*, 1996) and other bacteria. The HCP encoded by y3673 in the *Y. pestis* KIM locus y3658–y3677 was abundant in cytoplasmic and periplasmic fractions of cells grown to stationary phase at 26 °C in chemically defined media. Average 2DGE spot intensities were 4.8 ($n=3$; SD 1.2) in cytoplasmic and 4.5 ($n=3$; SD 0.6) in periplasmic fractions. The latter fraction is shown in one gel of Fig. 4(A) (Pp:26). The abundance of cytoplasmic and periplasmic HCP was at least 10-fold lower in cells isolated from the mid-exponential phase at 26 °C and from the stationary phase at 26 °C after growth in brain heart infusion broth. Apparently, the expression of HCP is regulated not only by temperature, but also by factors linked to population density and/or nutrient starvation.

To assess whether HCP is secreted by *Y. pestis* cells, this protein was profiled in 2DGE gels derived from CCS fractions. To assess whether HCP is potentially cell-surface-attached or released under proteolytic stress conditions, it was profiled in 2DGE gels derived from extracellular fractions after trypsin digestion of whole cells (td-Ext fractions). HCP spots were not detected in the CCS of cells grown at 37 °C or in the supernatant of cells incubated with trypsin for 15 h after growth at 37 °C (td-Ext:37, Fig. 4A). HCP was ninefold more abundant in the td-Ext fraction than in the CCS of cells grown at 26 °C. Average spot intensities were 8.2 ($n=3$; SD 0.9) and 0.9 ($n=3$; SD 0.4), respectively. The respective 2DGE spot patterns are displayed in Fig. 4(A). While it is plausible that HCP was actively secreted by the T6SS under these conditions, we could not rule out its release from cells permeabilized by prolonged treatment with trypsin. Most proteins visualized in the gels for trypsin-digested cell supernatants (td-Ext:26, td-Ext:37; Fig. 4A) were cytoplasmic (e.g. GroEL and AhpC) or periplasmic (e.g. MalE). A few proteins (e.g. GroEL) showed an abundance increase in the

Fig. 4. Distribution of HCP (y3673) in subcellular fractions and its oligomeric forms. (A) CBB-stained gel images represent a *Y. pestis* cell culture supernatant from cells grown at 26 °C (CCS:26), a periplasmic supernatant (Pp:26), and supernatants from trypsin-treated cells derived from growth temperatures of 37 and 26 °C (td-Ext:37, td-Ext:26). The proteins denoted in the 2DGE images are HCP (19 kDa), GroEL (57.5 kDa), AhpC (22.4 kDa) and MalE (43.8 kDa). (B) Samples, equivalent to those provided in (A) (gels on the right), were applied to an SEC column and eluted with the following median native M_r values (in kDa): lane 1, >500; lane 2, 400; lane 3, 220; lane 4, 125; lane 5, 45; lane 6, 20. In the CBB-stained SDS-PAGE gels, arrows indicate gel bands corresponding to the M_r of HCP. This was confirmed by MALDI-TOF analysis.

trypsin-digested cell supernatant, compared to the CCS fraction, at 26 °C that was comparable to that of HCP.

SEC experiments were performed to assess the native M_r value of HCP, a 19 kDa protein, in the supernatant of cells incubated with trypsin (26 °C), the cell lysate derived from these trypsin-treated cells, and the periplasmic fraction (26 °C). In the supernatant of cells incubated with trypsin, (extracellular) HCP was enriched in a native M_r range between ~150 and 100 kDa (lane 4, bottom gel; Fig. 4B). While this protein may form complexes with other proteins, a more rational explanation is a homo-oligomeric structure (five or six subunits). A hexameric ring structure has been attributed to HCP secreted by the *P. aeruginosa* T6SS (Mougous *et al.*, 2006). In the lysate and periplasmic fractions, *Y. pestis* HCP was identified by MS in SEC fractions corresponding to a broader M_r range (~150–30 kDa). As shown for the periplasmic fraction in Fig. 4(B) (lane 4, top gel), the abundance of HCP in the higher M_r range was low. HCP was also highly resistant to degradation by proteases, including trypsin. Fragments were not detected in cytoplasmic and periplasmic or in trypsin-treated cell supernatants. In summary, we have gained preliminary evidence that the T6SS forms a high- M_r protein complex in the IM, involving the proteins y3674 and y3675, and that an oligomeric HCP is released from *Y. pestis* cells following treatment with trypsin.

DISCUSSION

The *Y. pestis* OM proteome was shown to be highly dynamic, comparing growth temperatures that mimic the flea vector and mammalian host environments. Less information was gained on abundance changes in the IM proteome, to some extent due to the difficulty in profiling integral IM proteins in 2DGE gels. As previously reported (Han *et al.*, 2004; Hixson *et al.*, 2006; Motin *et al.*, 2004), the *Y. pestis* antigens CafI and KatY and the virulence-associated integral OM protease Pla were increased in abundance at 37 versus 26 °C. The abundance of Pla also increased when cells reached the stationary phase. Quorum sensing (Bobrov *et al.*, 2007) and the sigma factor RpoS (Miller & Bassler, 2001) may be involved in regulating *pla* expression. The Hms biofilm formation system was previously characterized as important for colonization and blocking of the flea proventriculus and transmission of *Y. pestis* from blocked fleas to rodents (Hinnebusch *et al.*, 1996; Jarrett *et al.*, 2004; Perry *et al.*, 2004). DNA microarray data have reported increased expression of *hms* genes at 26 versus 37 °C (Han *et al.*, 2004; Motin *et al.*, 2004). A more detailed analysis, however, has revealed post-transcriptional regulation of the Hms system, and a Lon- or ClpPX-dependent pathway for post-translational degradation of several Hms proteins was proposed (Perry *et al.*, 2004). In this study, two Hms proteins localized in the OM (HmsF and HmsH) were strongly increased at 26 versus 37 °C. Post-translational truncation products of HmsF and HmsH were detected in 2DGE gels as

reproducible spots, indicative of targeted proteolysis. These truncated proteins were of lower abundance than the full-length proteins and primarily detected at 26 °C. Thus, these data did not provide further evidence for selective post-translational degradation at 37 °C.

OM porins are required for small molecule transport across the OM. Strong abundance differences, e.g. for OmpC, OmpF and PhoE, at 26 versus 37 °C suggest that the transport of small molecules (ions, sugars, antibiotics) is highly regulated and tuned to the nutrient demands of *Y. pestis* in flea and host environments. Expression of *ompC* and *ompF* orthologues in *E. coli* (Pratt *et al.*, 1996) and *Serratia marcescens* (Begic & Worobec, 2006) is influenced by multiple environmental factors, such as ambient temperature, osmolarity and pH. The two *E. coli* porins are reciprocally regulated by a sophisticated mechanism in which the two-component regulator OmpR/EnvZ plays a central role (Yoshida *et al.*, 2006). A corresponding mode of regulation appears to occur in *Y. pestis*, supported by the detection of OmpR as a peripheral membrane protein in this survey.

Marked temperature-dependent changes in the abundance of several (putative) small β -barrel OM proteins were observed. The protein Ail (y1324) was the most abundant protein in the OM of *Y. pestis* KIM6+ at 37 °C, which may be crucial to its dual functional role in mediating bacterial resistance to complement-mediated killing and adherence to epithelial cells (Bartra *et al.*, 2008; Kolodziejek *et al.*, 2007). Ail was shown to be required for virulence in a *Caenorhabditis elegans* infection model, but not in an intravenous mouse model of the plague (Bartra *et al.*, 2008). While the *C. elegans* genome encodes a few putative complement components, a complement-mediated pathway facilitating innate immunity does not appear to exist. In contrast, cell-matrix adherence processes involving *C. elegans* hypodermal epithelial cells occur (Hong *et al.*, 2001). Confusingly, Ail and the protein y1682 are annotated as OmpX in the KIM genome. y1682, termed OmpX here, was strongly increased at 26 versus 37 °C. This was also reported in a recent proteomic analysis using the *Y. pestis* KIM5 strain (Hixson *et al.*, 2006). Opposite to Ail, OmpX lacks serum-protective activities and is not involved in biofilm-independent killing of *C. elegans* (Bartra *et al.*, 2008). We hypothesize that *ail* and *ompX* are reciprocally temperature-regulated at 26 versus 37 °C, analogous to OmpC and OmpF, and that OmpX has a specific functional role in the flea. Three additional putative small β -barrel OM proteins (y4083, y1795 and y2167) whose cellular localizations were unknown prior to this study were strongly increased at 37 °C. y2167 has a conserved domain that interacts with a membrane-bound lytic transglycosylase MltA and penicillin-binding protein 1B in *E. coli* (Vollmer *et al.*, 1999). No sequence similarities were denoted in annotations for y4083 and y1795. These proteins are interesting targets to elucidate functional roles in the host environment of *Y. pestis*.

The T6SS, a membrane-associated protein secretion apparatus expressed by many Gram-negative bacteria, has been implicated in the virulence of several human pathogens, but is structurally and functionally not well characterized (Dudley *et al.*, 2006; Mougous *et al.*, 2006; Nano *et al.*, 2004; Parsons & Heffron, 2005; Pukatzki *et al.*, 2006; Rao *et al.*, 2004; Schell *et al.*, 2007). Several aspects pertaining to key components of the T6SS have already been discussed in the Results. For the first time, we experimentally determined associations of a large number of putative T6SS proteins with membranes in a Gram-negative bacterium. In the *Y. pestis* KIM strain, all of these proteins were expressed from one of the five predicted T6SS gene clusters (y3658–y3677) and were not detectable in cells grown at 37 °C. A strong influence of temperature on y3658–y3677 gene expression was previously reported in transcriptional DNA microarray studies using two different *Y. pestis* strains (Han *et al.*, 2004; Motin *et al.*, 2004). Increased expression at 25 versus 37 °C was also reported for a T6SS of the fish pathogen *Ed. tarda* (Rao *et al.*, 2004; Zheng & Leung, 2007).

HCP (y3673) was characterized as a highly abundant, soluble intracellular protein in stationary-phase *Y. pestis* cells grown at 26 °C. The evidence for active secretion of this protein, which appears to be the main extracellular component of the T6SS and forms oligomeric structures (Mougous *et al.*, 2006), was ambiguous. HCP was barely detected in extracellular media of *Y. pestis* cell cultures at 26 °C, but highly abundant in extracellular fractions after prolonged treatment of *Y. pestis* cells with trypsin. The *Y. pestis* HCP isolated from such extracellular fractions appeared to assume homo-oligomeric states. The lack of HCP secretion has recently been linked to a resting state of the T6SS in *P. aeruginosa* (Mougous *et al.*, 2007). There was some evidence that trypsin treatment permeabilized *Y. pestis* cells, resulting in protein leakage, and induced a stress response. Like HCP, stress-response proteins such as GroEL and AhpC were highly abundant in the supernatants of trypsin-treated *Y. pestis* cells. Such proteins, particularly heat-shock proteins, have been linked to the formation of protein–lipid microdomains in *E. coli* membranes during cellular stress that, in turn, affects protein translocation through membranes (Horvath *et al.*, 2008). We speculate that complex regulatory mechanisms involving changes in population density and stress response levels are implicated in the release of HCP from *Y. pestis* cells via the T6SS. An altered membrane microdomain environment may facilitate the release of HCP. Further experiments examining links between cellular stress and HCP release are needed to support this notion. Interestingly, the temperature- and cell-density-dependent expression of HCP is reminiscent of bacterial virulence factors thought to be under control of global stress response regulators, such as RpoS and CpxA/R, and/or quorum sensing. Recently, expression of a T6SS of the plant pathogen *Pectobacterium atrosepticum* was shown to be influenced by quorum sensing (Liu *et al.*, 2008). In summary, we have demonstrated that the

expression of *Y. pestis* T6SS subunits is highly regulated and hypothesize that the T6SS plays a role during colonization of the flea vector.

Mutational analysis experiments for T6SS subunits have demonstrated that three proteins are essential for the secretion of HCP in *P. aeruginosa* (Mougous *et al.*, 2006, 2007), *V. cholerae* (Pukatzki *et al.*, 2006; Williams *et al.*, 1996), *Ed. tarda*, where HCP is termed EvpC (Zheng & Leung, 2007), and enteroaggregative *E. coli*, where HCP is termed AaiC (Dudley *et al.*, 2006). These proteins are a ClpB-type ATPase, a VasK-like integral membrane protein and an uncharacterized protein with the conserved COG domain 3517. The *Y. pestis* orthologue of the latter protein (y3674) has monotopic-integral IM protein traits. A full-length and an N-terminally truncated version of y3674 were enriched in a high- M_r protein fraction, suggesting self-association or complex formation with other T6SS subunits, such as y3675 and ClpB2. Further experiments are needed to elucidate structural features of the membrane-localized T6SS.

ACKNOWLEDGEMENTS

This work was performed under the Pathogen Functional Genomics Resource Center contract (contract no. N01-AI15447), funded by the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

REFERENCES

- Anisimov, A. P., Lindler, L. E. & Pier, G. B. (2004). Intraspecific diversity of *Yersinia pestis*. *Clin Microbiol Rev* **17**, 434–464.
- Bagos, P. G., Liakopoulos, T. D., Spyropoulos, I. C. & Hamodrakas, S. J. (2004). PRED-TMBB: a web server for predicting the topology of beta-barrel outer membrane proteins. *Nucleic Acids Res* **32**, W400–W404.
- Bartra, S. S., Styer, K. L., O'Bryant, D. M., Nilles, M. L., Hinnebusch, B. J., Aballay, A. & Plano, G. V. (2008). Resistance of *Yersinia pestis* to complement-dependent killing is mediated by the Ail outer membrane protein. *Infect Immun* **76**, 612–622.
- Begic, S. & Worobec, E. A. (2006). Regulation of *Serratia marcescens* *ompF* and *ompC* porin genes in response to osmotic stress, salicylate, temperature and pH. *Microbiology* **152**, 485–491.
- Bingle, L. E., Bailey, C. M. & Pallen, M. J. (2008). Type VI secretion: a beginner's guide. *Curr Opin Microbiol* **11**, 3–8.
- Black, P. N., Said, B., Ghosn, C. R., Beach, J. V. & Nunn, W. D. (1987). Purification and characterization of an outer membrane-bound protein involved in long-chain fatty acid transport in *Escherichia coli*. *J Biol Chem* **262**, 1412–1419.
- Bobrov, A. G., Bearden, S. W., Fetherston, J. D., Khweek, A. A., Parrish, K. D. & Perry, R. D. (2007). Functional quorum sensing systems affect biofilm formation and protein expression in *Yersinia pestis*. *Adv Exp Med Biol* **603**, 178–191.
- Brubaker, R. R. (2002). *Yersinia pestis*. In *Molecular Medical Microbiology*, pp. 2033–2058. Edited by M. Sussman. London, UK: Academic Press.
- Chain, P. S., Hu, P., Malfatti, S. A., Radnedge, L., Larimer, F., Vergez, L. M., Worsham, P., Chu, M. C. & Andersen, G. L. (2006). Complete

- genome sequence of *Yersinia pestis* strains Antiqua and Nepal516: evidence of gene reduction in an emerging pathogen. *J Bacteriol* **188**, 4453–4463.
- Chauvaux, S., Rosso, M. L., Frangeul, L., Lacroix, C., Labarre, L., Schiavo, A., Marceau, M., Dillies, M. A., Foulon, J. & other authors (2007). Transcriptome analysis of *Yersinia pestis* in human plasma: an approach for discovering bacterial genes involved in septicemic plague. *Microbiology* **153**, 3112–3124.
- Chromy, B. A., Choi, M. W., Murphy, G. A., Gonzales, A. D., Corzett, C. H., Chang, B. C., Fitch, J. P. & McCutchen-Maloney, S. L. (2005). Proteomic characterization of *Yersinia pestis* virulence. *J Bacteriol* **187**, 8172–8180.
- Cornelis, G. R. (2002). *Yersinia* type III secretion: send in the effectors. *J Cell Biol* **158**, 401–408.
- 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.
- Deng, W., Burland, V., Plunkett, G., III, Boutin, A., Mayhew, G. F., Liss, P., Perna, N. T., Rose, D. J., Mau, B. & other authors (2002). Genome sequence of *Yersinia pestis* KIM. *J Bacteriol* **184**, 4601–4611.
- Dudley, E. G., Thomson, N. R., Parkhill, J., Morin, N. P. & Nataro, J. P. (2006). Proteomic and microarray characterization of the AggR regulon identifies a *pheU* pathogenicity island in enteroaggregative *Escherichia coli*. *Mol Microbiol* **61**, 1267–1282.
- Fetherston, J. D. & Perry, R. D. (1994). The pigmentation locus of *Yersinia pestis* KIM6+ is flanked by an insertion sequence and includes the structural genes for pesticin sensitivity and HMWP2. *Mol Microbiol* **13**, 697–708.
- Fetherston, J. D., Schuetze, P. & Perry, R. D. (1992). Loss of the pigmentation phenotype in *Yersinia pestis* is due to the spontaneous deletion of 102 kb of chromosomal DNA which is flanked by a repetitive element. *Mol Microbiol* **6**, 2693–2704.
- Gatlin, C. L., Pieper, R., Huang, S. T., Mongodin, E., Gebregeorgis, E., Parmar, P. P., Clark, D. J., Alami, H., Papazisi, L. & other authors (2006). Proteomic profiling of cell envelope-associated proteins from *Staphylococcus aureus*. *Proteomics* **6**, 1530–1549.
- Han, Y., Zhou, D., Pang, X., Song, Y., Zhang, L., Bao, J., Tong, Z., Wang, J., Guo, Z. & other authors (2004). Microarray analysis of temperature-induced transcriptome of *Yersinia pestis*. *Microbiol Immunol* **48**, 791–805.
- Hinnebusch, B. J. (2004). The evolution of flea-borne transmission of *Yersinia pestis*. In *Yersinia Molecular and Cellular Biology*, pp. 49–73. Edited by E. Carniel & B. J. Hinnebusch. Wymondham, Norfolk, UK: Horizon Bioscience.
- Hinnebusch, B. J., Perry, R. D. & Schwan, T. G. (1996). Role of the *Yersinia pestis* hemin storage (*hms*) locus in the transmission of plague by fleas. *Science* **273**, 367–370.
- 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.
- Hixson, K. K., Adkins, J. N., Baker, S. E., Moore, R. J., Chromy, B. A., Smith, R. D., McCutchen-Maloney, S. L. & Lipton, M. S. (2006). Biomarker candidate identification in *Yersinia pestis* using organism-wide semiquantitative proteomics. *J Proteome Res* **5**, 3008–3017.
- Hong, L., Elbl, T., Ward, J., Franzini-Armstrong, C., Rybicka, K. K., Gatewood, B. K., Baillie, D. L. & Bucher, E. A. (2001). MUP-4 is a novel transmembrane protein with functions in epithelial cell adhesion in *Caenorhabditis elegans*. *J Cell Biol* **154**, 403–414.
- Horvath, I., Multhoff, G., Sonnleitner, A. & Vigh, L. (2008). Membrane-associated stress proteins: more than simply chaperones. *Biochim Biophys Acta* **1778**, 1653–1664.
- Hu, P., Elliott, J., McCready, P., Skowronski, E., Ganes, J., Kobayashi, A., Brubaker, R. R. & Garcia, E. (1998). Structural organization of virulence-associated plasmids of *Yersinia pestis*. *J Bacteriol* **180**, 5192–5202.
- Jarrett, C. O., Deak, E., Isherwood, K. E., Oyston, P. C., Fischer, E. R., Whitney, A. R., Kobayashi, S. D., DeLeo, F. R. & Hinnebusch, B. J. (2004). Transmission of *Yersinia pestis* from an infectious biofilm in the flea vector. *J Infect Dis* **190**, 783–792.
- Kolodziejek, A. M., Sinclair, D. J., Seo, K. S., Schnider, D. R., Deobald, C. F., Rohde, H. N., Viall, A. K., Minnich, S. S., Hovde, C. J. & other authors (2007). Phenotypic characterization of OmpX, an Ail homologue of *Yersinia pestis* KIM. *Microbiology* **153**, 2941–2951.
- Lillard, J. W., Jr, Bearden, S. W., Fetherston, J. D. & Perry, R. D. (1999). The haemin storage (*Hms*⁺) phenotype of *Yersinia pestis* is not essential for the pathogenesis of bubonic plague in mammals. *Microbiology* **145**, 197–209.
- Lindler, L. E., Plano, G. V., Burland, V., Mayhew, G. F. & Blattner, F. R. (1998). Complete DNA sequence and detailed analysis of the *Yersinia pestis* KIM5 plasmid encoding murine toxin and capsular antigen. *Infect Immun* **66**, 5731–5742.
- Liu, H., Coulthurst, S. J., Pritchard, L., Hedley, P. E., Ravensdale, M., Humphris, S., Burr, T., Takle, G., Brurberg, M. B. & other authors (2008). Quorum sensing coordinates brute force and stealth modes of infection in the plant pathogen *Pectobacterium atrosepticum*. *PLoS Pathog* **4**, e1000093.
- Lucier, T. S., Fetherston, J. D., Brubaker, R. R. & Perry, R. D. (1996). Iron uptake and iron-repressible polypeptides in *Yersinia pestis*. *Infect Immun* **64**, 3023–3031.
- Miller, M. B. & Bassler, B. L. (2001). Quorum sensing in bacteria. *Annu Rev Microbiol* **55**, 165–199.
- Molloy, M. P. (2008). Isolation of bacterial cell membranes proteins using carbonate extraction. *Methods Mol Biol* **424**, 397–401.
- Motin, V. L., Georgescu, A. M., Fitch, J. P., Gu, P. P., Nelson, D. O., Mabery, S. L., Garnham, J. B., Sokhansanj, B. A., Ott, L. L. & other authors (2004). Temporal global changes in gene expression during temperature transition in *Yersinia pestis*. *J Bacteriol* **186**, 6298–6305.
- Mougous, J. D., Cuff, M. E., Raunser, S., Shen, A., Zhou, M., Gifford, C. A., Goodman, A. L., Joachimiak, G., Ordonez, C. L. & other authors (2006). A virulence locus of *Pseudomonas aeruginosa* encodes a protein secretion apparatus. *Science* **312**, 1526–1530.
- Mougous, J. D., Gifford, C. A., Ramsdell, T. L. & Mekalanos, J. J. (2007). Threonine phosphorylation post-translationally regulates protein secretion in *Pseudomonas aeruginosa*. *Nat Cell Biol* **9**, 797–803.
- Myers-Morales, T., Cowan, C., Gray, M. E., Wulff, C. R., Parker, C. E., Borchers, C. H. & Straley, S. C. (2007). A surface-focused biotinylation procedure identifies the *Yersinia pestis* catalase KatY as a membrane-associated but non-surface-located protein. *Appl Environ Microbiol* **73**, 5750–5759.
- Nano, F. E., Zhang, N., Cowley, S. C., Klose, K. E., Cheung, K. K., Roberts, M. J., Ludu, J. S., Letendre, G. W., Meierovics, A. I. & other authors (2004). A *Francisella tularensis* pathogenicity island required for intramacrophage growth. *J Bacteriol* **186**, 6430–6436.
- Parkhill, J., Wren, B. W., Thomson, N. R., Titball, R. W., Holden, M. T., Prentice, M. B., Sebahia, M., James, K. D., Churcher, C. & other

- authors (2001).** Genome sequence of *Yersinia pestis*, the causative agent of plague. *Nature* **413**, 523–527.
- Parsons, D. A. & Heffron, F. (2005).** *sciS*, an *icmF* homolog in *Salmonella enterica* serovar Typhimurium, limits intracellular replication and decreases virulence. *Infect Immun* **73**, 4338–4345.
- Perry, R. D. & Fetherston, J. D. (1997).** *Yersinia pestis* – etiologic agent of plague. *Clin Microbiol Rev* **10**, 35–66.
- Perry, R. D. & Fetherston, J. D. (2004).** Iron and heme uptake systems. In *Yersinia Molecular and Cellular Biology*, pp. 257–283. Edited by E. Carniel & B. J. Hinnebusch. Wymondham, Norfolk, UK: Horizon Bioscience.
- Perry, R. D., Bobrov, A. G., Kirillina, O., Jones, H. A., Pedersen, L., Abney, J. & Fetherston, J. D. (2004).** Temperature regulation of the hemin storage (Hms⁺) phenotype of *Yersinia pestis* is posttranscriptional. *J Bacteriol* **186**, 1638–1647.
- Pieper, R., Huang, S. T., Clark, D. J., Robinson, J. M., Parmar, P. P., Alami, H., Bunai, C. L., Perry, R. D., Fleischmann, R. D. & Peterson, S. N. (2008).** Characterizing the dynamic nature of the *Yersinia pestis* periplasmic proteome in response to nutrient exhaustion and temperature change. *Proteomics* **8**, 1442–1458.
- Pratt, L. A., Hsing, W., Gibson, K. E. & Silhavy, T. J. (1996).** From acids to *osmZ*: multiple factors influence synthesis of the OmpF and OmpC porins in *Escherichia coli*. *Mol Microbiol* **20**, 911–917.
- Pukatzki, S., Ma, A. T., Sturtevant, D., Krastins, B., Sarracino, D., Nelson, W. C., Heidelberg, J. F. & Mekalanos, J. J. (2006).** Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the *Dictyostelium* host model system. *Proc Natl Acad Sci U S A* **103**, 1528–1533.
- Pukatzki, S., Ma, A. T., Revel, A. T., Sturtevant, D. & Mekalanos, J. J. (2007).** Type VI secretion system translocates a phage tail spike-like protein into target cells where it cross-links actin. *Proc Natl Acad Sci U S A* **104**, 15508–15513.
- Rao, P. S., Yamada, Y., Tan, Y. P. & Leung, K. Y. (2004).** Use of proteomics to identify novel virulence determinants that are required for *Edwardsiella tarda* pathogenesis. *Mol Microbiol* **53**, 573–586.
- Schell, M. A., Ulrich, R. L., Ribot, W. J., Brueggemann, E. E., Hines, H. B., Chen, D., Lipscomb, L., Kim, H. S., Mrazek, J. & other authors (2007).** Type VI secretion is a major virulence determinant in *Burkholderia mallei*. *Mol Microbiol* **64**, 1466–1485.
- Sodeinde, O. A., Subrahmanyam, Y. V., Stark, K., Quan, T., Bao, Y. & Goguen, J. D. (1992).** A surface protease and the invasive character of plague. *Science* **258**, 1004–1007.
- Song, Y., Tong, Z., Wang, J., Wang, L., Guo, Z., Han, Y., Zhang, J., Pei, D., Zhou, D. & other authors (2004).** Complete genome sequence of *Yersinia pestis* strain 91001, an isolate avirulent to humans. *DNA Res* **11**, 179–197.
- Vollmer, W., von Rechenberg, M. & Holtje, J. V. (1999).** Demonstration of molecular interactions between the murein polymerase PBP1B, the lytic transglycosylase MltA, and the scaffolding protein MipA of *Escherichia coli*. *J Biol Chem* **274**, 6726–6734.
- Williams, S. G., Varcoe, L. T., Attridge, S. R. & Manning, P. A. (1996).** *Vibrio cholerae* Hcp, a secreted protein coregulated with HlyA. *Infect Immun* **64**, 283–289.
- Wren, B. W. (2003).** The yersiniae – a model genus to study the rapid evolution of bacterial pathogens. *Nat Rev Microbiol* **1**, 55–64.
- Yoshida, T., Qin, L., Egger, L. A. & Inouye, M. (2006).** Transcription regulation of *ompF* and *ompC* by a single transcription factor, OmpR. *J Biol Chem* **281**, 17114–17123.
- Zavialov, A. V., Berglund, J., Pudney, A. F., Fooks, L. J., Ibrahim, T. M., MacIntyre, S. & Knight, S. D. (2003).** Structure and biogenesis of the capsular F1 antigen from *Yersinia pestis*: preserved folding energy drives fiber formation. *Cell* **113**, 587–596.
- Zheng, J. & Leung, K. Y. (2007).** Dissection of a type VI secretion system in *Edwardsiella tarda*. *Mol Microbiol* **66**, 1192–1206.
- Zhou, D., Han, Y., Song, Y., Huang, P. & Yang, R. (2004a).** Comparative and evolutionary genomics of *Yersinia pestis*. *Microbes Infect* **6**, 1226–1234.
- Zhou, D., Tong, Z., Song, Y., Han, Y., Pei, D., Pang, X., Zhai, J., Li, M., Cui, B. & other authors (2004b).** Genetics of metabolic variations between *Yersinia pestis* biovars and the proposal of a new biovar, microtus. *J Bacteriol* **186**, 5147–5152.

Edited by: G. Thomas