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

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*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 Ms 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.*

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).

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 Ca2+ 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 haemins storage system (Hms)-dependent biofilm system. Biofilm formation allows colonization of the flea proventriculus, causing blocking 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 phase transition 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, assemblies 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 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-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 lysosome/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 30 000 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 study, 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 about 15 h at 4°C. The supernatants, extracted at this high pH and termed hpH-MBR fractions, were concentrated 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 ush-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.

**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 thermoregulated proteins. 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 P₃-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; CafI, #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-acetylglucosaminyl-1-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).
| Spot no. | Accession no. | Gene locus† | Gene name‡ | Subcell. cat.§ | Protein name and description | 26 vs 37 °C low Pi || | 26 vs 37 °C low Fe§ | 26 vs 37 °C usb-MBR# | 26 vs 37 °C hs-MBR** |
|----------|--------------|-------------|-------------|---------------|----------------------------|------------------------|---------------|------------------------|--------------------------|------------------------|
| 1        | 22123952     | y0031       | malK        | IM            | ATP-binding protein of maltose/maltodextrin ABC transporter | –1.7                   |               |                        |                          |                        |
| 2        | 22123953     | y0032       | lamB        | OM            | Maltoporin                      | –10                    | –3.1          |                        |                          |                        |
| 3        | 22124056     | y0136       | (yhcB)      | IM            | Cytochrome d ubiquinol oxidase subunit III                  | 2.3                    | 1.9           | 1.5                    |                          |                        |
| 4        | 22124057     | y0137       | degQ        | pM            | Serine endoprotease                  | 2.3                    | 4             | 2.5                    |                          |                        |
| 5        | 22124081     | y0161       | pyrB        | pM            | Aspartate carbamoyltransferase catalytic subunit            | 6.7                    | 4.7           | 2.9                    |                          |                        |
| 6        | 22124122     | y0202       | mreB        | pM            | Regulator of FtsI/penicillin-binding protein 3               | 3.1                    |               |                        |                          |                        |
| 7        | 22124311     | y0396       | pldA        | OM            | OM phospholipase A                                  | –4                     | –3.4          | –6.3                   |                          |                        |
| 8        | 22124523     | y0609       | groEL       | MSL           | Chaperonin GroEL                      | 6.7                    | 7             | 3.1                    |                          |                        |
| 9        | 22124636     | y0722       | phnM        | pM            | Phosphonate metabolism protein                   | –5                     |               |                        |                          |                        |
| 10       | 22124731     | y0818       | cysJ        | pM            | NADPH-dependent sulfite reductase, flavoprotein β-subunit | –10                   | –10          | –5                     |                          |                        |
| 11       | 22124761     | y0850       | (cirA2)     | OM            | Putative TonB-dependent OM receptor for iron transport     | 3.2                    | –6.3          |                        |                          |                        |
| 12       | 22124781     | y0870       | katY        | MSL           | Catalase [hydroperoxidase HPI(I)]                      | 2.6                    | 2.6           | 6.3                    |                          |                        |
| 13       | 31795399     | Y1069       | ynt         | Cy            | Yersinia murine toxin                              | –2.2                   | –4.8          | –3.8                   | –1.7                    |                        |
| 14       | 22125123     | y1221       | proV        | IM            | ATP-binding subunit of glycine/betaine/proline transporter | –1.7                   | –2.3          | –1.9                    |                          |                        |
| 15       | 22125223     | y1324       | ail          | OM            | Attachment invasion locus protein Ail                  | 6.7†                   | >1.5, n.s.v. | 6.9†                   | >1.5, n.s.v. |                        |
| 16       | 22125261     | y1362       | guaB        | pM            | Inositol-5-monophosphate dehydrogenase                  | –2.6                   | –7.8          | –3.3                    |                          |                        |
| 17       | 22125317     | y1419       | nlpB        | OM            | Lipoprotein NlpB                            | >1.5, n.s.v. | >1.5, n.s.v. |                        |                          |                        |
| 18       | 22125472     | y1577       | fidL        | OM            | Long-chain fatty acid OM transporter                  | 3.8                    |               |                        |                          |                        |
| 19       | 22125474     | y1579       | fidI        | pM            | 3-Ketoacyl-CoA thiolase                               | 3.8                    | 5.1           |                        |                          |                        |
| 20       | 22125577     | y1682       | ompX        | 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 N-acetylmuramoyl-1-alanine amidase; regulator | –3.5                   | 2.6           |                        |                          |                        |
| 23       | 22125812     | y1919       | (wcaG9)     | M             | Bifunctional UDP-glucuronic acid decarboxylase/formyltransferase | –2.3                   |               |                        |                          |                        |
| 24       | 22125993     | y2104       | (ydgA)      | pM            | Putative phospholipid-binding lipoprotein y2104          | –1.6                   | –2.1          |                        |                          |                        |
| 25       | 22126055     | y2167       | (ompV)      | OM            | Hypothetical protein; putative OM protein V            | 1.9                    | 1.8, 6.4†††     |                        |                          |                        |
| 26       | 22126242     | y2358       | hmsF        | OM            | Haemin storage/biofilm formation protein HmsF           | –2.2                   | –2             | –2.7                    |                          |                        |
| 27       | 22126243     | y2359       | hmsH        | OM            | Haemin storage/biofilm formation protein HmsH           | –8.1                   | –4.2           | –5.2, <−10§§         |                          |                        |
| 28       | 22126278     | y2394       | ybtS        | pM            | Salicylate synthase Irp9 (yersiniabactin biosynthesis) | –1.5                   |               |                        |                          |                        |
| 29       | 22126286     | y2402       | ybtT        | pM            | Yersiniabactin thiosterase                          | –1.5                   |               |                        |                          |                        |
| 30       | 22126287     | y2403       | ybtE        | pM            | Salicyl-AMP ligase (yersiniabactin biosynthesis)          | –2.1                   |               |                        |                          |                        |
| 31       | 22126288     | y2404       | psn         | OM            | Pesticin/yersiniabactin OM receptor                  | –4                     |               |                        |                          |                        |
| 32       | 22126437     | y2556       | fcuA        | OM            | TonB-dependent OM ferrichrome receptor                 | 2.1                    | 4.8           |                        |                          |                        |
| 33       | 22126613     | y2735       | ompA        | OM            | OM protein A                                   | –1.6, –3, –3.3†††       |               |                        |                          |                        |
| 34       | 22126637     | y2759       | ompF        | OM            | OM porin OmpF                                | –10                   | –4.3          | –10                    | –14.3                   |                        |
| 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       | clpA       | pM             | ATP-binding subunit of serine protease | 3.0             |                 |                 |                  |                 |
| 36        | 22126843        | y2966       | ompC       | OM             | OM porin OmpC            | 3.1             | 3.4             | 2.6             | 7.5              |                 |
| 37        | 22126860        | y2983       | phoE       | OM             | OM phosphoprotein     | −4.8            |                 |                 |                  |                 |
| 38        | 22126930        | y3054       | pal        | OM             | Peptidoglycan-associated OM lipoprotein | <−10, n.s.v.    |                 | −6.3, n.s.v.    |                  |                 |
| 39        | 22127183        | y3007       | fba        | Cy             | Fructose-bisphosphate aldolase | 4.9             | −2.7             | 6.4             | 1.5              |                 |
| 40        | 22127186        | y3310       | tktA       | 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       | secA       | pM             | Preprotein translocase subunit SecA | 4               | −3              |                 | −2.2             |                 |
| 44        | 22127543        | y3669       | clpB2      | 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, −6.3‡‡    |                 |                 |
| 47        | 22127549        | y3675       | –          | pM             | Putative T6SS subunit y3675 | <−10            | <−10            | <−10            |                  |                 |
| 48        | 22127730        | y3859       | uspA       | pM             | Universal stress protein | 1.7             |                 |                 | 1.5              |                 |
| 49        | 22127786        | y3916       | ompR       | pM             | Osmolarity response regulator OmpR | 4.8             | 2.7             |                 |                  |                  |
| 50        | 22127855        | y3985       | fusA       | MSL            | GTP-binding protein chain-elongation factor (EF-G) | 2, >10‡‡        | 4.8, >10‡‡      |                 |                  |                  |
| 51        | 22127856        | y3986       | tufB       | 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       | pstB       | IM             | ATP-binding protein of high-affinity phosphate ABC transporter | −2.1            |                 |                 |                  |                  |
| 54        | 31795234         | YPKMT065    | cafI       | OM             | F1 capsule antigen    | >10             | >10             | 13.9            |                  |                  |
| 55        | 31795332         | YPKp077     | pla        | 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).
‡ 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).
§ 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).
|| Urea/amidosulfobetaine-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/amidosulfobetaine-extracted membrane fraction, 37 °C vs 26 °C (negative value when increased at 26 °C); cells were grown in iron-depleted PMH2 medium.
## Urea/amidosulfobetaine-extracted membrane fraction, 37 °C vs 26 °C (negative value when increased at 26 °C); cells were grown in 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, low-phosphate PMH2 medium (0.12 M K$_2$HPO$_4$); usb-MBR, -Fe, PMH2 medium depleted of iron (FeCl$_3$); 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 Mv 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 Pi-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 Pi-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 Pi-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 Pi-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 Mv 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. pl 2DGE | Obs. Mᵣ 2DGE | Predict. pl¶ | Predict. Mᵣ¶ | N-terminal peptide# | Mascot score** |
|-----------------|-----------|---------------|----------------------|--------------------------------------------------------------------------------|-----------------|----------------|----------------|-----------------|----------------|------------------|-----------------|
| 22127532        | y3658     | PA0077        | y IM                | VasK/IcmF-like integral membrane protein                                       | 3523            | 5.9ₚ            | 33ₚ            | 9               | 133            | N₁₂₄−R₁₈₄        | 85              |
| 22127533        | y3659     | PA0078        | y IM                | VasF-like integral membrane protein                                            | 3455            | 6.7ₚ            | 46ₚ            | 8.6             | 62             | L₂₇₁−K₇₃₄         | 27              |
| 22127534        | y3660     | PA0079        | ? M                  | Integral membrane protein                                                       | –               | 5.3            | 45             | 5.6             | 51             | M₁−K₁₀₀          | 0_TOF           |
| 22127537        | y3663     | –             | ? Ex                 | Putative secreted protein                                                       | –               | 6.3            | 23             | 8               | 22             | T₃₄−K₇₉         | 20              |
| 22127538        | y3664     | –             | ? M                  | Integral membrane protein with pentapeptide repeats                             | 1357            | 5.7            | 43             | 5.7             | 42             | M₁−R₁₅          | 61              |
| 22127539        | y3665     | –             | ? M                  | Integral membrane protein with pentapeptide repeats                             | 1357            | 5.5            | 86             | 5.4             | 85             | L₂₃₁−R₂₅₁        | 20              |
| 22127540        | y3668     | PA0095        | y M                  | VgrG-like integral membrane protein                                             | 3501            | 5.2            | 98             | 5.2             | 86             | V₅₆−R₆₇         | 27              |
| 22127543        | y3669     | PA0090        | y pIM                | ATP-dependent protease ClpB2                                                    | 542             | 5.7            | 97             | 5.6             | 97             | T₃₃−R₂₉         | 46              |
| 22127545        | y3671     | PA0088        | y M                  | VasA-like integral membrane protein                                             | 3519            | 6.1            | 69             | 6.3             | 69             | V₄₁₇−R₁₂₇        | 69              |
| 22127547        | y3673     | PA0085        | y C, P, Ex           | Secreted haemolysin-coregulated protein HCP                                     | 3157            | 5.2            | 18             | 5.2             | 19             | W₂₂−K₅₂         | 50              |
| 22127548        | y3674     | PA0084        | y mIM                | Putative monotopic IM protein                                                   | 3517            | 5.3            | 60             | 5.3             | 57             | A₇−K₉₉          | 92              |
| 22127549        | y3674     | PA0084        | y mIM                | Post-translational variant of y3674                                            | 3517            | 5.6ₚ            | 54ₚ            |                |                | R₄₆−K₁₆₆        | 69              |
| 22127550        | y3676     | PA0082        | y M                  | ImpA-like integral membrane protein                                             | 3515            | 4.7            | 41             | 4.8             | 39             | L₆₅−R₇₃         | 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.
¶ Mᵣ and pl values predicted from gi| annotations.
# Most N-terminal peptides identified by LC-MS/MS.
**MS/MS score for N-terminal peptides (Mascot data).
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

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**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 Na_2CO_3 (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.

**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 Na_2CO_3 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.

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507

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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 \( ^\circ \)C in chemically defined media. Average 2DGE spot intensities were 4.8 \((n=3; \text{SD} 1.2)\) in cytoplasmic and 4.5 \((n=3; \text{SD} 0.6)\) 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 \( ^\circ \)C and from the stationary phase at 26 \( ^\circ \)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 \( ^\circ \)C or in the supernatant of cells incubated with trypsin for 15 h after growth at 37 \( ^\circ \)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 \( ^\circ \)C. Average spot intensities were 8.2 \((n=3; \text{SD} 0.9)\) and 0.9 \((n=3; \text{SD} 0.4)\), 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

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**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 \( ^\circ \)C (CCS:26), a periplasmic supernatant (Pp:26), and supernatants from trypsin-treated cells derived from growth temperatures of 37 and 26 \( ^\circ \)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; Hixon et al., 2006; Motin et al., 2004), the Y. pestis antigens Caf1 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 (Hixon 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 MlaA 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 enterohaemorrhagic 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-Mr, 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.

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