Contributions of chaperone/usher systems to cell binding, biofilm formation and *Yersinia pestis* virulence

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*Yersinia pestis* genome sequencing projects have revealed six intact uncharacterized chaperone/usher systems with the potential to play roles in plague pathogenesis. We cloned each locus and expressed them in the Δ*flim* *Escherichia coli* strain AAEC185 to test the assembled *Y. pestis* surface structures for various activities. Expression of each chaperone/usher locus gave rise to specific novel fibrillar structures on the surface of *E. coli*. One locus, *y0561-0563*, was able to mediate attachment to human epithelial cells (HEp-2) and human macrophages (THP-1) but not mouse macrophages (RAW264.7), while several loci were able to facilitate *E. coli* biofilm formation. When each chaperone/usher locus was deleted in *Y. pestis*, only deletion of the previously described pH 6 antigen (Psa) chaperone/usher system resulted in decreased adhesion and biofilm formation. Quantitative RT-PCR (qRT-PCR) revealed low expression levels for each novel chaperone/usher system *in vitro* as well as in mouse tissues following intravenous infection. However, a *Y. pestis* mutant in the chaperone/usher locus *y1858-1862* was attenuated for virulence in mice via the intravenous route of infection, suggesting that expression of this locus is, at some stage, sufficient to affect the outcome of a plague infection. qRT-PCR experiments also indicated that expression of the chaperone/usher-dependent capsule locus, caf1, was influenced by oxygen availability and that the well-described chaperone/usher-dependent pilus, Psa, was strongly induced in minimal medium even at 28 °C rather than 37 °C, a temperature previously believed to be required for Psa expression. These data indicate several potential roles for the novel chaperone/usher systems of *Y. pestis* in pathogenesis and infection-related functions such as cell adhesion and biofilm formation.

INTRODUCTION

*Yersinia pestis*, the causative agent of plague, is believed to have evolved from *Yersinia pseudotuberculosis* 1500–20 000 years ago (Achtman *et al.*, 1999). Plague is a devastating infectious disease that has decimated populations throughout history (Cantor, 2001; Perry & Fetherston, 1997). While all pathogenic *Yersinia* species carry the 70 kb Yop-encoding virulence plasmid (pCD1 in *Y. pestis*), *Y. pestis* carries two additional plasmids that contribute to its flea-transmitted lifestyle: pMT1 (encoding the F1 capsule and several other factors) and pPCP1 (encoding pesticin, pesticin immunity and plasminogen activator). Six different *Yersinia* outer proteins (Yops) have been described in *Y. pestis* and these are translocated into host cells via a pCD1-encoded type III secretion system. Yops inhibit phagocytosis by disrupting the host cell cytoskeleton, blocking the pro-inflammatory cytokine response or inducing apoptosis of macrophages (Cornelis *et al.*, 1998; Cornelis, 2002).

While a clear role for Yop-mediated cytotoxicity and plasminogen activator-dependent dissemination of an infection from the site of flea inoculation have been
described (Beesley et al., 1967; Goguen et al., 1986; Higuchi & Smith, 1961; Sodeinde et al., 1992; Straley & Gibull, 1989; Straley & Bowmer, 1986; Welkos et al., 1997), we hypothesized that other \textit{Y. pestis} surface proteins may be important during a plague infection.

Chaperone/usher systems often mediate assembly of fimbrial surface structures that confer adhesive and pathogenic capacity to bacteria (Hoschützky et al., 1989; Kuehn et al., 1992; Thanassi et al., 1998). Chaperone/usher-associated fimbrial subunits cross the inner membrane of Gram-negative bacteria via the Sec secretion system. Fimbrial subunits that eventually make up the surface structure are bound in the periplasm by a co-expressed chaperone molecule. The region of the fimbrial surface structure are bound in the periplasm by a co-expression outer-membraneusher-associated fimbrial subunits cross the inner membrane of Gram-negative bacteria via the Sec secretion system. Fimbrial subunits that eventually make up the surface structure are bound in the periplasm by a co-expressed chaperone molecule. The region of the fimbrial subunit bound by the chaperone is composed of an immunoglobulin-like \(\beta\)-sheet fold that is missing one \(\beta\)-strand. The chaperone molecule binds to the fimbrial subunit destined for secretion by providing the missing \(\beta\)-strand in a process known as donor-strand complementation (Barnhart et al., 2000; Choudhury et al., 1999; Sauer et al., 1999). The chaperone then delivers the monomeric fimbrial subunit to a co-expressed outer-membrane usher protein. The usher protein functions as an assembly and secretion platform, catalysing polymerization of the subunit proteins into fimbriae (pili) and providing the channel for secretion of the fimbriae across the outer-membrane to the cell surface (Nishiyama et al., 2008; Remaut et al., 2008). Thus, each chaperone/usher system is composed of a collection of genes encoding one or multiple chaperone proteins, an usher protein and one or more secreted subunit proteins destined for secretion to the surface of the bacterium.

Upon sequencing of two \textit{Y. pestis} strains, KIM10 + [biovar Mediaevalis (Deng et al., 2002)] and CO92 [biovar orientalis (Parkhill et al., 2001)], eight potentially functional chaperone/usher systems were identified. To date, two chaperone/usher systems have been well studied in \textit{Y. pestis}: Caf1 (F1) capsule and pH 6 antigen (Psa). The Caf1 capsule has antiphagocytic activity against macrophages in tissue culture and it is an excellent protective antigen against bubonic plague, especially when combined with V antigen (LcrV) (Anderson et al., 1996; DeBord et al., 2006; Zavialov et al., 2003). However, the capsule appears to be dispensable for \textit{Y. pestis} virulence (Drozdov et al., 1995; Quenee et al., 2008). In fact, it was recently observed that \textit{Y. pestis} escape mutants can spontaneously arise in mice immunized with Caf1 antigen due to IS1541 element insertion within the caf1 locus (Cornelius et al., 2009). Thus, relying on anti-Caf1 antibodies for protection against \textit{Y. pestis} infections is not advisable. The other well-studied chaperone/usher system in \textit{Y. pestis} mediates elaboration of Psa on the surface of the bacterium (Ben-Efraim et al., 1961). Psa carries out a number of activities, including haemagglutination of red blood cells from several animal species (Bichowsky-Slomnicki & Ben-Efraim, 1963; Lindler & Tall, 1993), adhesion to cultured cells (Yang et al., 1996) and macrophages (Makoveichuk et al., 2003), and binding to the Fc region of human IgG (Zav’yalov et al., 1996). Psa has also been shown to have antiphagocytic activity for macrophages, similar to the Caf1 capsule (Huang & Lindler, 2004). Disruption of the Psa locus results in attenuation of \textit{Y. pestis} infection via the intravenous route of inoculation (Felek et al., 2010; Lindler et al., 1990), indicating a role in pathogenesis for this antiphagocytic adhesin.

Given the important activities of both Caf1 and Psa during host infection, we anticipated that other chaperone/usher systems encoded within \textit{Y. pestis} may also play roles in virulence. These roles could include adhesion, invasion, biofilm formation, antiphagocytosis or other activities. This study investigates functions and expression patterns of the six novel chaperone/usher loci (Fig. 1), using Psa as a positive control for some known activities.

We demonstrate that the structure encoded by locus y0561-0563 mediates adhesion of recombinant \textit{Escherichia coli} to human epithelial and macrophage-like cells, but not

\textbf{Fig. 1.} Chaperone/usher systems in the \textit{Y. pestis} KIM genome investigated in this study (Deng et al., 2002). White arrows, putative fimbrial (secreted) proteins; grey arrows, chaperones; black arrows, usher proteins; hatched arrows, large potentially secreted proteins.
murine macrophages. In addition, four novel chaperone/usher loci of *Y. pestis* facilitate *E. coli* biofilm formation. Finally, deletion of the *Y. pestis y1858-1662* locus results in a virulence defect following intravenous inoculation of mice.

**METHODS**

**Bacterial strains and plasmids.** Characteristics and sources of the bacterial strains and plasmids used in this study are given in Supplementary Table S1 (available with the online version of this paper). Gene deletions in *Y. pestis* KIM5 were constructed by using PCR products as described previously (O'Toole & DePaola, 2000; Felek et al., 2008; Yu et al., 2000). For cloning, the genes encoding chaperone/usher systems were amplified by PCR. PCR products and plasmid pMMB207 were cut by using appropriate enzymes, purified by gel purification and ligated into plasmid pMMB207. Because strain KIM5 is *pgn*−, it does not have the y2388-2392 locus (Buchrieser et al., 1999). We cloned this locus from *Y. pseudotuberculosis* strain IP2666. *Y. pestis* KIM and *Y. pseudotuberculosis* IP2666 have 100% base sequence identity for effector proteins of this system. *E. coli* DH5α was transformed with the ligation products and chloramphenicol-resistant colonies were selected. Clones were confirmed by DNA sequencing.

**SDS-PAGE.** *E. coli* AAEC185 strains were cultured overnight in LB medium supplemented with 10 µg chloramphenicol ml−1 and 100 µM IPTG at 28 ºC and outer-membrane preparations were obtained as described previously (Hantke, 1984). Isolated outer-membranes were subjected to 15% SDS-PAGE followed by Coomassie blue staining. Whole-cell lysates and outer-membrane preparations were also run on SDS-PAGE and transferred to nitrocellulose membrane for Western blotting using a 1:1000 dilution of an anti-RNA polymerase α-subunit antibody (Neoclone) to verify that the outer membrane preparations were free of cytosolic contents as described previously (Felek et al., 2008).

**Preparation of *E. coli* cells for electron microscopy (EM).** *E. coli* cultures were inoculated from single colonies, grown overnight at 30 ºC, diluted 1:40 into fresh medium, and then grown at 37 ºC for ~2 h to OD600 0.8 (exponential phase). IPTG was added to 50 µM final concentration to induce expression of the chaperone/usher pathways. For transmission EM, the bacteria were harvested, washed once with PBS, adsorbed onto polyvinyl formal-carbon-coated grids (Ernest F. Fullam) for 2 min, and fixed with 1% glutaraldehyde (Sigma-Aldrich) for 15 min at room temperature. Wells were washed three times with PBS. Crystal violet (0.01%) was added and cells were stained for 15 min at room temperature. Wells were washed three times with distilled water and crystal violet was solubilized with 80% ethanol and 20% acetic mixture. The optical density of the mixture was detected by using a microplate reader at 595 nm. Digital images were acquired with an AMT XR-60 CCD digital camera system (Advanced Microscopy Techniques).

**Adhesion assays.** Twenty-four-well cell culture plates of HEp-2 and RAW264.7 cells were prepared at ≥80% confluence as described previously (Felek et al., 2008). THP-1 cells were cultured and prepared for infection as described previously (Felek & Krukonis, 2009), with the alteration that after 3 days differentiation in the presence of the phorbol ester 12-myristate 13-acetate, THP-1 were removed from a T75 flask by trypsinization and plated into 24-well plates overnight prior to infection.

*E. coli* AAEC185 strains containing pMMB207- or pMMB207-encoded chaperone/usher systems were cultured overnight at 28 ºC with shaking in Luria-Bertani (LB) medium supplemented with 10 µg chloramphenicol ml−1 and 100 µM IPTG. Since some recombinant proteins were toxic for *E. coli* AAEC185 at 37 ºC under inducing conditions, strains were cultured at 28 ºC. Bacterial cultures were centrifuged and resuspended in MEM for HEp-2 cells, DMEM for RAW264.7 cells or RPMI 1640 for THP-1 cells. Host cells were infected with bacteria at a m.o.i. of about 1–3 bacteria per cell after the host cells were washed twice with MEM or DMEM lacking serum. RAW264.7 cells and THP-1 cells were treated with 5 µg cytochalasin-D ml−1 for 45 min to paralyse macrophages before the addition of bacteria. Plates were incubated at 37 ºC, 5% CO2 for 2 h and wells were washed twice with PBS. Cells were lysed with 500 µl distilled water +0.1% Triton X-100 (Bio-Rad) for 10 min at room temperature and samples were saved. Wells were washed once more with PBS and washes were saved. Some wells were not washed to remove unbound bacteria in order to calculate total bacteria per well. For this purpose, all media and washes were saved. Ten-fold dilutions in PBS were plated on LB agar plates containing 10 µg chloramphenicol ml−1. The plates were incubated at 37 ºC overnight and the output c.f.u. were enumerated.

For *Yersinia* adhesion, *Y. pestis* KIM5, KIM5 pCD1− and various KIM5 deletion mutants were cultured overnight at 28 ºC pH 7, 37 ºC pH 7 or 37 ºC pH 6 in heart infusion broth (HIB), with shaking. Cultures were diluted to OD520 0.15 in HIB pH 7.0 and incubated for 4 h under the same culture conditions with shaking. Cultures were pelleted and resuspended in tissue culture medium without serum to OD520 1.5. Cells were infected at an m.o.i. of 1–3 bacteria per host cell for 2 h as for the *E. coli* binding assays. Binding efficiency was assessed by a c.f.u. plating assay as described previously (Felek & Krukonis, 2009).

**Biofilm assays.** Crystal violet staining was used to detect cells attached to polystyrene as described previously (O’Toole et al., 1999). Briefly, overnight cultures were diluted 1:100 into LB plus 10 µg chloramphenicol ml−1 and 100 µM IPTG in flat-bottom polystyrene culture plates (Costar Corning) and incubated for 24 h at 28 ºC without shaking. The OD95 of cultures was read by using a microplate reader (Perkin Elmer Lambda Reader). Plates were washed three times with PBS. Crystal violet (0.01%) was added and cells were stained for 15 min at room temperature. Wells were washed three times with distilled water and crystal violet was solubilized with 80% ethanol and 20% acetic mixture. The optical density of the mixture was detected by using a microplate reader at 595 nm. Results were normalized with the OD95 of crystal violet/bacterial density of AAEC185+pMMB207 set equal to 1.0. For *Y. pestis* biofilms, KIM5 derivatives were grown in PMH2 minimal medium (Gong et al., 2001) at 28 ºC pH 7, 37 ºC pH 7 and 37 ºC pH 6 and processed as described for *E. coli*. For *Y. pestis*, raw data for OD95/bacterial density are presented without normalization.

**Mouse experiments.** Female Swiss Webster mice (6–8-weeks-old) were obtained from Harlan Sprague–Dawley. *Y. pestis* strains were cultured overnight at 28 ºC in HIB pH 7, pelleted and resuspended in PBS to approximately 500 bacteria ml−1. Each mouse was intravenously inoculated with 100 µl of this suspension. At the same time, c.f.u. assays were performed to determine the inoculum c.f.u. for each group. Measured inocula varied from 50 to 56 bacteria per mouse for all KIM5 derivatives except for the KIM5 pCD1-negative strain, for which we used 10 400 bacteria per mouse. Mice were checked daily for survival and signs of illness. Statistical differences in survival between groups were assessed using the logrank test. Animal experiments were performed according to approvals provided by the University of Michigan UCUCA office.

**Quantitative RT-PCR (qRT-PCR) expression calculations.** To determine the expression of various KIM5 loci relative to the gene encoding the RNA polymerase β subunit under various growth
conditions, Y. pestis mRNA was isolated as described previously *in vitro* (Felek et al., 2008) or following homogenization of harvested tissues. mRNA (500 ng) was converted to cDNA using Superscript II RT (Invitrogen) and 5 ng cDNA was used for 50 cycles of qRT-PCR using a Bio-Rad iCycler. Once the cycles at which detectable qRT-PCR signals were determined, the mean cycle at which *rpoB* was detected was calculated and the cycle at which each other locus was detected (*n*=5–6 measurements over two experiments) was subtracted from the mean detection cycle for *rpoB*. This gave the number of cycles difference in detection between *rpoB* and a locus of interest. If this difference was positive, the locus was expressed to higher levels than *rpoB* (detected at an earlier qRT-PCR cycle number). To measure the fold increase in expression, the number of cycles difference, *X*, was used as a power function in the equation 2^*X*; since each cycle of qRT-PCR represents a doubling of the PCR targets. Using this equation, a locus that was detected five cycles prior to *rpoB* would be expressed 2^5=32 times higher relative to *rpoB*. The signal from qRT-PCR in the absence of reverse transcriptase was subtracted and standard deviations of the expression measurements were calculated. Primers for qRT-PCR analysis are listed in Supplementary Table S2.

**RESULTS**

**Expression of chaperone/usher system proteins in the outer membrane**

We took two approaches to visualize expression of the various chaperone/usher system proteins from *Y. pestis* in *E. coli*. First, we prepared outer-membrane extracts from *E. coli* strain AAEC185 (*Δfim*) expressing each of the six novel *Y. pestis* chaperone/usher loci from the IPTG-inducible plasmid pMMB207 (Morales et al., 1991). Outer membranes would be expected to contain the usher protein from each system and the secreted protein(s) if the secreted protein is not shed from the surface during preparation of the membranes, a common issue for fimbriae and pili (Felek et al., 2008; Hoschützky et al., 1989)]. Coomassie staining of outer-membrane preparations from AAEC185 demonstrated that clearly visible novel proteins are expressed from two of six loci (Fig. 2; *y0561-0563* and *y1869-1873*). The novel bands are of the predicted size for each anticipated secreted protein and are labelled with an asterisk (Fig. 2; Supplementary Table S3, available with the online version of this paper). Some chaperone/usher loci contain multiple putative secreted proteins. However, in all but one of these cases, one of the putative secreted proteins is between 38 and 48.5 kDa (*y0349, Y1872, Y2391* and *Y3478*, Supplementary Table S3) and thus is potentially obscured by the major outer-membrane proteins of *E. coli*. Chaperone/usher systems typically direct assembly of pili or fimbriae on the bacterial surface and these structures are often fragile (Hoschützky et al., 1989; Kuehn et al., 1992; Thanassi et al., 1998). In fact, shearing off from the surface due to centrifugation has been demonstrated for the *Y. pestis* chaperone/usher-dependent adhesin Psa (Felek et al., 2008). For this reason, we also looked at supernatants from AAEC185 strains expressing each of the *Y. pestis* chaperone/usher loci. We noticed an 18 kDa protein associated with the *y1869-1873* locus that was released into the culture supernatants (data not shown) and this protein was confirmed as the protein encoded by the *y1869* gene by mass spectrometry (data not shown). No other chaperone/usher-dependent surface appendage appeared to accumulate at high levels in the culture supernatants except Psa, as demonstrated previously (Felek et al., 2008). It is possible that several of the chaperone/usher-encoded pili are resistant to boiling in SDS and failed to enter the gel (Barnhart & Chapman, 2006; Thanassi & Hultgren, 2000). For all of the chaperone/ usher systems, we were unable to distinguish the 87–96 kDa usher protein from other endogenous *E. coli* outer-membrane proteins in a similar size range.

In addition to preparing outer membranes of *E. coli* to determine chaperone/usher system expression levels, we also prepared *E. coli* cells for EM. We anticipated that each novel chaperone/usher *Y. pestis* locus would result in elaboration of a particular structure on the surface of *E. coli*. All six novel chaperone/usher loci encode chaperone proteins predicted to have a short loop between β-strands

**Fig. 2.** Expression of chaperone/usher proteins in the outer membrane of *E. coli* AAEC185. Strains were cultured overnight under inducing conditions. Outer membranes were prepared and analysed by using Coomassie-blue-stained SDS-PAGE (a). Antibody demonstrated that the outer-membrane preparations were not contaminated with cytosolic contents: outer membranes (b) or whole-cell lysate (c). As a positive control on the outer-membrane protein Western blot (b), we included one lane of AAEC185+pMMB207 whole-cell extract (pMMB207 whole). Western blot lanes are aligned with lanes in the Coomassie gel. Target bands are labelled with asterisks. Predicted sizes: Y0561, 21.8 kDa; Y1869, 18.3 kDa.
F1–G1 and lack disulfide bond-forming cysteines within those strands. These features are indicative of chaperone/usher systems that assemble rigid pili (Hung et al., 1996; Runco et al., 2008). For the EM analysis, bacteria were grown overnight at 30 °C and then subcultured into fresh medium and grown at 37 °C with 50 μM IPTG to induce expression of the chaperone/usher pathways (Methods). Cells were prepared for EM and compared with AAEC185 induced with the empty vector pMMB207. The negative control strain AAEC185 + pMMB207 expressed thick appendages on its surface indicative of flagella expression (Fig. 3a). However, strains expressing the various Y. pestis chaperone/usher loci elaborated additional distinct structures on their surface. Expression of locus y0348-0352 resulted in the assembly of a small number of elongated fimbriae-like structures on the surface of E. coli (Fig. 3b). Locus y0561-0563 also expressed thin fimbriae-like filaments (Fig. 3c). Note the difference in thickness between the flagellum and the y0561-0563-encoded fimbriae-like structures (Fig. 3c). Expression of the y3478-3480 locus resulted in long filaments that were polarly localized in many instances (Fig. 3b). Expression of locus y1858-1862 resulted in the bacteria being coated with abundant, thin, peritrichous fimbriae (Fig. 3d). The neighbouring locus, y1869-1873, led to expression of a large number of thick, tangled filaments on the surface of E. coli (Fig. 3e); some bacteria expressing this locus produced twisted, rope-like filaments (Fig. 3f). Finally, expression of locus y2388-2392 resulted in assembly of a smaller number of thick fibres on the bacterial surface (Fig. 3g). This locus is encoded within the horizontally acquired high pathogenicity region of the Y. pestis chromosome (Buchrieser et al., 1999; Hare et al., 1999).

**Adhesion to cultured cells**

Since many chaperone/usher systems encode adhesive structures (Proft & Baker, 2009; Sauer et al., 2000), we tested the ability of each Y. pestis-encoded structure, when expressed in Δfim E. coli, to adhere to a number of different cell lines including human epithelial cells (HEp-2), human macrophage-like cells (THP-1) and mouse macrophages (RAW264.7 cells). Plate-grown bacteria were used in these studies to prevent shearing of pili from the bacterial cell during broth shaking. Expression of the y0561-0563 locus resulted in a three- or twofold increase in adhesion to HEp-2 (Fig. 4a) and THP-1 (Fig. 4b) cells, respectively, but conferred no increased adhesion to RAW264.7 cells (Fig. 4c). Thus, locus y0561-0563 may encode a surface appendage with adhesive activity specific for human cells. Our positive control for these experiments was the chaperone/usher-encoded surface adhesin, Psa (Galván et al., 2007; Lindler et al., 1990; Payne et al., 1998; Yang & Isberg, 1997).

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**Fig. 3.** EM images of E. coli AAEC185 (Δfim) expressing various Y. pestis chaperone/usher loci. Bacteria were induced for expression of the chaperone/usher loci at 37 °C with 50 μM IPTG. Arrows indicate structures of interest. In AAEC185 + pMMB207 (a), the arrow points to a flagellum; in the remaining panels, arrows point to novel fimbrial structures. Bars, 500 nm. 

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Expression of Psa resulted in a 45-fold increase in HEp-2 cell binding, a fourfold increase in THP-1 cell binding and a fivefold increase in binding to RAW264.7 cells compared with E. coli harbouring pMMB207 alone (Fig. 4).

**Fig. 4.** Adhesion of E. coli AAEC185 expressing novel chaperone/usher systems or Psa to HEp-2 (a), THP-1 (b) and RAW264.7 macrophage (c) cells. Strains were cultured overnight at 28 °C on LB agar plates supplemented with 10 µg chloramphenicol ml⁻¹ and 100 µM IPTG. Bacteria were added to tissue culture cells at an m.o.i. of 1–3. Phagocytosis of THP-1 and RAW264.7 cells was inhibited with 5 µg cytochalasin-D ml⁻¹ prior to addition of E. coli. Adhesion was determined by comparing the number of cell-associated c.f.u. with the total number of c.f.u. in the whole tissue culture well. Values are the means ±s.d. of three independent experiments with duplicate assays from each experiment. *P<0.05; **P<0.0005.

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**Adhesion of Y. pestis to cultured cells**

Y. pestis and related pathogenic *Versinia* species are known to produce a number of adhesins on their surface (Bliska *et al.*, 1993; El Tahir & Skurnik, 2001; Felek & Krukonis, 2009; Isberg *et al.*, 1987; Kolodziejek *et al.*, 2007; Lähteenmäki *et al.*, 1998; Miller & Falkow, 1988). This redundancy may allow for specific tissue tropism during infection, allow for fine tuned regulation during the progression of an infection, or serve as a backup mechanism to ensure efficient colonization and Yop delivery to host target cells. Although redundancy often obscures the loss of adhesive activity following deletion of a single adhesin, we constructed deletion strains of each novel *Y. pestis* chaperone/usher locus and the *psaA* locus and tested those strains for a role in *Y. pestis* adhesion to various cell lines. None of the novel chaperone/usher loci deletion mutants had a defect in HEp-2, THP-1 or RAW264.7 cell binding (Fig. 5), including a deletion of locus y0561, which was shown to encode an adhesin in *E. coli* (Fig. 4). For these studies, bacterial cells were grown under three different conditions to mimic different natural environments: 28 °C pH 7 (flea-like), 37 °C pH 7 (host infection) or 37 °C pH 6 [acidified tissues or within a phagolysosome, an environment known to induce Psa (Lindler & Tall, 1993)]. This indicates that other adhesins in addition to y0561 mediate *Y. pestis* adhesion in KIM5. Alternatively, locus y0561-0563 may not be expressed under the conditions tested. The Psa mutant (ΔpsaA) had an adhesion defect to all three cell lines tested when bacteria were grown at 37 °C pH 6. Thus, under these conditions, Psa was required for maximal binding as expected (Fig. 5).

**Role of chaperone/usher loci in biofilm formation**

Another function of some chaperone/usher-encoded structures is to mediate biofilm formation. Biofilm formation can play a role in colonization as well as resistance to innate killing mechanisms of the host (Fux *et al.*, 2005; Jefferson, 2004; Patel, 2005; Stewart & William Costerton, 2001). Each chaperone/usher locus was expressed in the Δ* fim* *E. coli* strain AAEC185 and early stage biofilm formation was assessed by the development of deposits in polystyrene 96-well plates. For these experiments, we tested biofilm formation at 28 °C (a temperature known to be important for *Y. pestis* biofilm formation in the flea gut) as well as at 37 °C, to investigate potentially novel roles for biofilms of *Y. pestis* during human infections.

At 28 °C, expression of three novel *Y. pestis* chaperone/usher loci (y0348-0352, y0561-0563 and y1858-1862) resulted in an increase in *E. coli* biofilm formation ranging from a 2- to 11-fold (Fig. 6). In addition, the previously characterized *psaABC* locus was able to mediate early stages of biofilm formation, a newly described function of Psa. When biofilms were allowed to form at 37 °C, locus y0561-0563 was again able to enhance *E. coli* biofilm formation, as was y3478-3480 (Fig. 6). Thus, many of the structures seen in Fig. 2 are able to facilitate early biofilm formation in *E. coli*. These may also be important either in the flea vector or during human infection. In these experiments, all loci were expressed from an inducible promoter to allow controlled expression. Related to this point, the ability of Psa to enhance biofilm formation was diminished at 37 °C (the temperature at which *psaABC* is expressed in *Y. pestis*). It is unclear why *E. coli* shows this preference, but as described below, Psa is important for *Y. pestis* biofilm formation under normal (37 °C pH 6) expression conditions.

**Role of chaperone/usher systems in hms-independent biofilms in *Y. pestis***

Biofilm formation in *Y. pestis* is primarily dependent upon genes of the *hms* system, including the *hmsHFRS* genes
within the pgm (pigmentation) locus and hmsP and hmsT outside of the pgm locus, that facilitate biofilm formation at ambient temperatures in fleas (Bobrov et al., 2008; Fetherston et al., 1992; Hinnebusch et al., 1996; Kirillina et al., 2004). The KIM5 strain lacks the 102 kb pgm locus due to spontaneous deletion via recombination of two IS100 elements (Fetherston et al., 1992). However, Y. pestis has also been shown to mediate hms-independent biofilm formation (Styer et al., 2005).

Given the ability of several Y. pestis chaperone/usher loci to facilitate E. coli biofilm formation (Fig. 6), we assessed the effect of each deletion on hms-independent biofilm formation in the pgm-negative strain KIM5. Deletion of each individual novel chaperone/usher system had no effect on KIM5 biofilm formation under three different growth conditions tested (Fig. 7). However, deletion of psaA led to a sixfold decrease in KIM5 biofilm formation when cells were grown at 37 °C pH 6 (Fig. 7c). This is the first report to our knowledge of biofilm-forming activity for Psa in Y. pestis.

Expression of chaperone/usher loci in vitro and in vivo

One reason that deletion of novel chaperone/usher loci of Y. pestis had no effect on certain phenotypes may be that each individual novel chaperone/usher system had no effect on KIM5 biofilm formation under three different growth conditions tested (Fig. 7). However, deletion of psaA led to a sixfold decrease in KIM5 biofilm formation when cells were grown at 37 °C pH 6 (Fig. 7c). This is the first report to our knowledge of biofilm-forming activity for Psa in Y. pestis.
usher-dependent F1 protein capsule of rpoB be expressed at 37\degree C overnight and expression levels were compared with the locus of interest was not expressed under that particular growth condition. qRT-PCR was used to assess expression levels of the various chaperone/usher systems under several conditions. Expression under three typical in vitro growth conditions was assessed, including HIB medium at 28 \degree C pH 7 (a), 37 \degree C pH 7 (b) or 37 \degree C pH 6 (c) and processed as described for E. coli. For Y. pestis, raw data for OD_{590}/bacterial density are presented without normalization. *P=0.00001.

As we were unable to detect robust expression of the five novel chaperone/usher systems of KIM5 in standard in vitro conditions, we also assessed expression in oxygen-limited conditions or in minimal medium. To produce microaerophilic conditions, bacteria were grown at 37 \degree C overnight in a sealed jar without shaking. Under these conditions, none of the novel chaperone/usher systems was expressed to levels higher than those found under aerated conditions previously (Fig. 8 and Supplementary Table S4). However, two unexpected observations were made regarding the well-studied caf1 and psaA loci. Under microaerophilic conditions at 37 \degree C, caf1 was only modestly expressed, at levels 3.9-fold above rpoB, compared with expression that was 405-fold above rpoB when shaken at 37 \degree C pH 7 (Fig. 8 and Supplementary Table S4). Thus, oxygen tension strongly affects caf1 expression. Also, while in some cases psaA was poorly expressed under microaerophilic conditions (Fig. 8 and Supplementary Table S4), in some experiments, psaA was expressed as high as 36-fold above rpoB, presumably depending upon whether the cells grown in microaerophilic conditions resulted in extensive acidification of the medium (data not shown).

Growth in minimal medium was another condition we explored to assess expression of novel chaperone/usher systems. PMH3 medium, a Y. pestis minimal medium based on PMH2 (Gong et al., 2001), supplemented with 25 mM HEPES (to stabilize the pH), was used after adjusting the starting pH to pH 7.5 or pH 6.0 as indicated (Methods). In PMH3, the novel chaperone/usher locus y0561 was expressed at levels approaching that of rpoB (0.33 relative expression) when grown at 28 \degree C and a starting pH of 7.5. The only other novel chaperone/usher locus expressed to levels approaching rpoB was y0352, which was expressed at relative levels between 0.22 and 0.32 under all three conditions tested in PMH3. All other novel chaperone/usher loci were expressed at levels <0.1 (Fig. 8 and Supplementary Table S4). Unexpectedly, caf1 was not very strongly expressed in PMH3 at 37 \degree C (Fig. 8 and Supplementary Table S4), a temperature known to induce caf1 in rich media (Fig. 8; Fox & Higuchi, 1958). Furthermore, upon growth in PMH3, psaA was altered in its regulation. Rather than being maximally activated at 37 \degree C pH 6, psaA was strongly activated at all three growth conditions in PMH3 (Fig. 8 and Supplementary Table S4). Over the course of overnight growth, all three cultures were acidified, but the 28 \degree C pH 7.5 culture was only mildly acidic at pH 6.6 at the end of the experiment, while the 37 \degree C pH 7.5 and 37 \degree C pH 6 cultures had final pH readings of 5.8 and 4.6, respectively. These data suggest that either some component of rich media represses psaA expression unless it is grown at 37 \degree C and pH <6.7 or a component of the minimal medium PMH3 induces expression of the psaABC locus, even at 28 \degree C. In any event, this result clearly indicates that psaA can be expressed to slightly higher levels than rpoB under all three standard in vitro conditions.
expressed under unconventional growth conditions depending on the medium composition.

Finally, as we were interested in assessing the role of the novel chaperone/ usher systems during infection, we assessed the expression levels of each locus from spleen and lung tissue after a 3 day mouse infection with 10 LD$_{50}$ (100 organisms) of KIM5 administered intravenously. In infected spleen tissue, three loci, $y0352$, $y1860$ and $y1869$, were expressed 15- to 100-fold higher compared with the same loci expressed in the rich medium, HIB (Fig. 8 and Supplementary Table S4), although their absolute expression levels were still modest: 0.12–0.30 relative to $rpoB$. Two other loci, $y0561$ and $y3478$ were undetectable or poorly expressed, respectively (Fig. 8 and Supplementary Table S4).

In lung tissue, four of the novel chaperone/usher loci were expressed to levels of 0.068 or below, relative to $rpoB$, while

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**Fig. 8.** qRT-PCR analysis of chaperone/ usher system expression in vitro and in vivo. KIM5 was grown in HIB (a), PMH-3 (b) or harvested from infected tissue 3 days post-infection (c). Expression was normalized to $rpoB$ and plotted on a log scale after subtracting the signal obtained from no reverse transcriptase controls. Transcript levels were also compared to transcripts for gapA and 16S rRNA. qRT-PCR assays were performed twice in duplicate or triplicate ($n$=5–6). nd, No amplification detected after 50 cycles of qRT-PCR.
y1869 was expressed to nearly the same level as rpoB, with relative expression of 0.59 (Fig. 8 and Supplementary Table S4). Even at their highest expression levels in tissues, none of the novel chaperone/usher loci reached the level of expression of rpoB, suggesting that these systems are expressed to modest levels or only a subpopulation induces expression. As expected, caf1 was strongly expressed in vivo. While psaA was expressed to intermediate levels in the spleen (fivefold above rpoB), it was not detected in lung tissue, as has been described previously (Lathem et al., 2005).

Virulence defects of chaperone/usher mutants

Given the potential roles of the Y. pestis chaperone/usher systems in tissue adhesion, biofilm formation or other virulence-associated activities, we determined whether there was any virulence defect associated with deletion of each Y. pestis chaperone/usher locus. Y. pestis KIM5 and various deletion mutants were tested in a mouse model of plague. Groups of 20 female Swiss Webster mice received 7 LD₅₀ (~50 bacteria (Felek et al., 2010)) intravenously. Mice were monitored for 14 days to determine whether they succumbed to the infection and a time-course survival curve was generated. While all mice infected with the parental strain (KIM5) had 0% survival by day 10, one chaperone/usher mutant, Δy1858-1862, showed a statistically significant increase in survival (Fig. 9, P=0.005). Deletion of y0561 also led to 10% survival; however, this increase was not statistically significant (P=0.33). All mice survived infection with a KIM5 pCD1⁻ strain (lacking the Yop-encoding plasmid) at a dose of 10 400 c.f.u.

Somewhat surprisingly, the ΔpsaA mutant had no defect in virulence (although one mouse did survive infection). This conflicts with the previous report of a similar mutant in KIM5 that was shown to be attenuated for virulence, with a 200-fold increase in LD₅₀ (Lindler et al., 1990). We also observed that the KIM5 ΔpsaA mutant was more strongly attenuated in mice in some experiments, but this defect appeared to be variable (Felek et al., 2010). This may reflect the acidity of the inoculating culture and whether Psa is expressed at the time of injection.

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**Fig. 9.** Mouse survival assay for Y. pestis KIM5 and individual deletion mutants. Overnight cultures of Y. pestis KIM5, KIM5 pCD1⁻ and deletion mutants were intravenously inoculated into mice at a dose of 5–10 LD₅₀ (~50 bacteria). Mice were observed for 14 days after injection for survival. Ten mice were infected per day and the experiment was repeated once. Data are combined from the two experiments. Solid line, survival curve for KIM5; dashed line, survival curve of chaperone/usher mutant (given above each plot). P values were determined using logrank tests.
DISCUSSION

Chaperone/usher systems allow for secretion of bacterial surface appendages that can provide various functions to the cell (Proft & Baker, 2009; Sauer et al., 2000). The goal of this study was to identify activities associated with six uncharacterized chaperone/usher systems of Y. pestis, assess their expression patterns and determine their roles in Y. pestis virulence. We found that, of the six novel chaperone/usher systems, y0561-0563 conferred adhesive activity to E. coli (Fig. 4), although deletion of y0561 had no effect on Y. pestis cell attachment (Fig. 5). Given that Y. pestis has several other adhesins including Ail (Felek & Krukonis, 2009; Kolodziejek et al., 2007), plasminogen activator (Pla; Kienle et al., 1992; Lähteenmäki et al., 1998) and Psa (Felek et al., 2008; Galván et al., 2007; Lindler et al., 1990; Yang & Isberg, 1997), we assume they (and other potential adhesins) may mask any binding defect of the y0561 deletion mutant. Some novel Y. pestis chaperone/usher systems had a slight inhibitory effect on adhesion when expressed in E. coli (Fig. 4b). Based on EM of E. coli expressing these structures, at least two novel chaperone/usher pathways, y1858-1862 and y1869-1873, may abundantly coat the bacterium with pilus-like structures potentially occluding other surface adhesins (Fig. 3d and e).

Biofilms can be important virulence factors for bacteria. They can help facilitate colonization and protect bacteria from immune defences of the host (Fux et al., 2005; Jefferson, 2004; Patel, 2005; Stewart & William Costerton, 2001). Attachment of Gram-negative bacteria to a surface via outer-membrane proteins is the first step of biofilm formation, followed by replication to form microcolonies and produce a mature biofilm. In E. coli we found several Y. pestis chaperone/usher systems were able to facilitate biofilm formation upon expression. Among the two growth conditions tested (28 and 37 °C), four novel chaperone/usher systems could enhance E. coli biofilms (Fig. 6). The well-studied chaperone/usher-dependent adhesin Psa was also able to enhance E. coli biofilms. Given that the y0561-0563 locus confers both adhesive and biofilm-forming activities to E. coli, we considered the possibility that strains expressing y0561-0563 are non-specifically sticky to all surfaces. However, we found that E. coli expressing y0561-0563 does not autoaggregate due to bacterial–bacterial interactions over a 6 h time-course, whereas expression of the previously described Y. pestis autotransporter, YapC, led to robust autoaggregation (50 % settling out of solution within 90 min, data not shown) as demonstrated previously (Felek et al., 2008).

Although the Y. pestis strain used in these studies, KIM5, lacks the major biofilm-forming locus (hmsHFRS within the larger pgm locus), we were able to assess the ability of various chaperone/usher system mutants to form previously reported hms-independent biofilms (Styer et al., 2005). Deletion of each individual novel chaperone/usher system had no effect on Y. pestis hms-independent biofilm formation. However, deletion of psaA led to a sixfold decrease in hms-independent biofilms of Y. pestis when the bacteria were grown at a starting pH of 6.0. This is the first report of Psa contributing to biofilm formation in Y. pestis. This activity adds to the growing list of Psa functions, including binding to β1-linked galactosyl residues in glycosphingolipids (Payne et al., 1998) and to phosphatidylcholine (Galván et al., 2007), interaction with plasma lipoproteins (Makoveichuk et al., 2003), acting as an Fc-receptor for human IgG (Zav’yalov et al., 1996) and blocking phagocytosis (Huang & Lindler, 2004).

We also constructed chaperone/usher deletion mutants of Y. pestis KIM6+, a strain fully competent for biofilm formation (Darby et al., 2002; Hinnebusch et al., 1996). Previous studies have shown that KIM6+ derivatives defective for biofilm formation are unable to block Caenorhabditis elegans from feeding on Y. pestis, leading to ingestion of the bacteria and C. elegans development to mature forms (Darby et al., 2002). None of the KIM6+ derivatives deleted for any of the six novel chaperone/usher loci (nor a ΔpsaA mutant) were defective for blockage of C. elegans feeding (Creg Darby, personal communication). qRT-PCR analysis was used to assess expression levels of the various chaperone/usher systems under a variety of in vitro and in vivo conditions. While a few conditions gave modest levels of expression of some loci – y0352 (spleen and minimal medium), y0561 (minimal medium), y1860 (spleen) and y1869 (spleen and lung) – most conditions tested showed low expression levels (<0.1) of the novel chaperone/usher loci relative to the housekeeping gene rpoB. However, the well-characterized chaperone/usher systems controlling F1 capsule (caf1) and Psa (psaA) production were well expressed under conditions shown previously to induce their expression, 37 °C and 37 °C pH 6, respectively. During our extensive analysis we observed some conditions where caf1 would be predicted to be expressed to high levels (various 37 °C conditions) but was not. These included poor expression of caf1 at 37 °C in microaerophilic conditions (perhaps mimicking the low oxygen tension in non-vascularized necrotic lesions) and in minimal medium (Fig. 8 and Supplementary Table S4). Growth in minimal medium also gave unanticipated results concerning psaA expression. Rather than no expression at 28 °C starting pH 7.5, this condition resulted in a high level of expression of psaA (1000-fold above rpoB; Fig. 8 and Supplementary Table S4). Whereas in rich medium, growing a culture at 37 °C and a starting pH of 6.0 gave the highest levels of psaA expression, in minimal medium, this gave the lowest levels of expression, although psaA was still strongly expressed (450-fold above rpoB; Fig. 8 and Supplementary Table S4).

The level of psaA expression in pH 6 PMH-3 medium at 37 °C was significantly different from psaA expression in pH 7 medium at 37 °C (P=0.011), but not pH 7 medium at 28 °C (P=0.096). As psaA is at least partially regulated by the global regulator rovA in Y. pestis (Cathelyn et al., 2006), these changes may reflect effects on rovA expression or RovA activity. Alternatively, these conditions may affect...
the expression or activity of PsAE, a membrane-bound activator of psaA that is encoded immediately upstream of psaA along with a periplasmically located effector protein PsAF (Yang & Isberg, 1997). While psaA has been shown previously to be induced within phagolysosomes of macrophages in a pH-dependent fashion (requiring acidification; Lindler & Tall, 1993), it remains to be determined whether psaA expression in other tissues may reflect the minimal medium situation and not be as strongly dependent upon acidic pH (Fig. 8 and Supplementary Table S4).

Our study demonstrated that deletion of one novel chaperone/usher locus, y1858-1862, led to decreased virulence in mice after intravenous infection, indicating that it has a role in plague pathogenesis (Fig. 9). This locus was able to facilitate biofilm formation in E. coli (Fig. 6) and also interfere with E. coli adhesion to THP-1 (macrophage-like) cells (Fig. 4b). Expression of this locus in E. coli also resulted in abundant, peritrichous fibriniae on the surface of the bacterium. All of these activities may contribute to the role of this locus in plague pathogenesis.

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We thank Dr James Bliska for supplying Y. pseudotuberculosis strain IP2666, as a source of the y2388-2392 chaperone/ usher locus. This locus was able to facilitate biofilm formation in E. coli (Fig. 6) and also interfere with E. coli adhesion to THP-1 (macrophage-like) cells (Fig. 4b). Expression of this locus in E. coli also resulted in abundant, peritrichous fibriniae on the surface of the bacterium. All of these activities may contribute to the role of this locus in plague pathogenesis.

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