

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 Δfim *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.

Received 19 August 2010
Revised 11 November 2010
Accepted 16 November 2010

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

Abbreviations: EM, electron microscopy; qRT-PCR, quantitative RT-PCR.

Four supplementary tables are available with the online version of this paper.

described (Beesley *et al.*, 1967; Goguen *et al.*, 1986; Higuchi & Smith, 1961; Sodeinde *et al.*, 1992; Straley & Cibull, 1989; Straley & Bowmer, 1986; Welkos *et al.*, 1997), we hypothesized that other *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 subunit bound by the chaperone is composed of an immunoglobulin-like β -sheet fold that is missing one β -strand. The chaperone molecule binds to the fimbrial subunit destined for secretion by providing the missing β -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 *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 *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 *Y. pestis* virulence (Drozdov *et al.*, 1995; Quenee *et al.*, 2008). In fact, it was recently observed that *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 *Y. pestis* infections is not advisable. The other well-studied chaperone/usher system in *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 *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 *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 *Escherichia coli* to human epithelial and macrophage-like cells, but not

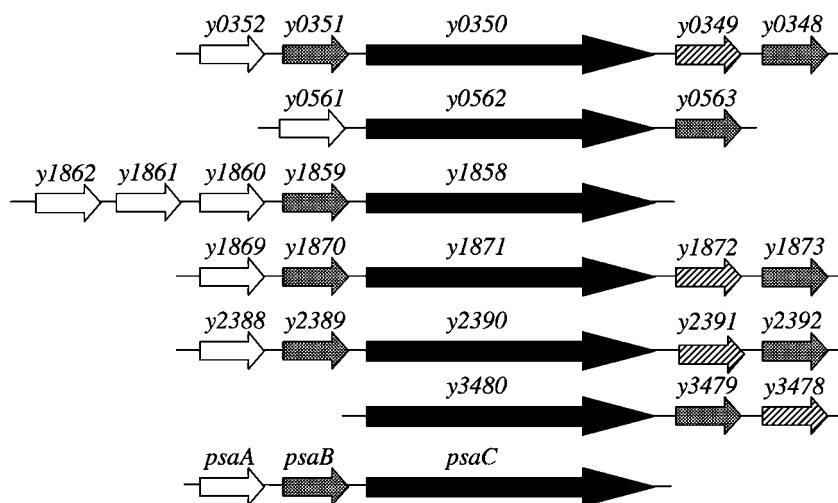


Fig. 1. Chaperone/usher systems in the *Y. pestis* KIM genome investigated in this study (Deng *et al.*, 2002). White arrows, putative fimbrial (secreted) proteins; grey arrows, chaperones; black arrows, usher proteins; hatched arrows, large potentially secreted proteins.

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 (Datsenko & Wanner, 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 *pgm*⁻, 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* DH5 α 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 μ g chloramphenicol ml⁻¹ and 100 μ M IPTG at 28 °C 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 °C, diluted 1:40 into fresh medium, and then grown at 37 °C for ~2 h to OD₆₀₀ 0.8 (exponential phase). IPTG was added to 50 μ M 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 1 min. The grids were washed twice with PBS and twice with water, and then negatively stained with 0.5% phosphotungstic acid (Ted Pella) for 35 s. The grids containing the negatively stained bacteria were examined on an FEI TECNAI BioTwin G⁰² microscope (FEI) at 80 kV accelerating voltage. 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 \geq 80% confluency as described previously (Felek *et al.*, 2008). THP-1 cells were cultured and prepared for infection as described previously (Felek & Krukoniš, 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 °C with shaking in Luria-Bertani (LB) medium supplemented with

10 μ g chloramphenicol ml⁻¹ and 100 μ M IPTG. Since some recombinant proteins were toxic for *E. coli* AAEC185 at 37 °C under inducing conditions, strains were cultured at 28 °C. 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 an 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 μ g cytochalasin-D ml⁻¹ for 45 min to paralyze macrophages before the addition of bacteria. Plates were incubated at 37 °C, 5% CO₂ for 2 h and wells were washed twice with PBS. Cells were lysed with 500 μ l double 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 μ g chloramphenicol ml⁻¹. The plates were incubated at 37 °C 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 °C pH 7, 37 °C pH 7 or 37 °C pH 6 in heart infusion broth (HIB), with shaking. Cultures were diluted to OD₆₂₀ 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 OD₆₂₀ 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 & Krukoniš, 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 μ g chloramphenicol ml⁻¹ and 100 μ M IPTG in flat-bottom polystyrene culture plates (Costar Corning) and incubated for 24 h at 28 °C without shaking. The OD₅₉₅ 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% acetone mixture. The optical density of the mixture was detected by using a microplate reader at 595 nm. Results were normalized with the OD₅₉₅ 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 °C pH 7, 37 °C pH 7 and 37 °C pH 6 and processed as described for *E. coli*. For *Y. pestis*, raw data for OD₅₉₅/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 °C in HIB pH 7, pelleted and resuspended in PBS to approximately 500 bacteria ml⁻¹. Each mouse was intravenously inoculated with 100 μ l 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 UCUC 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

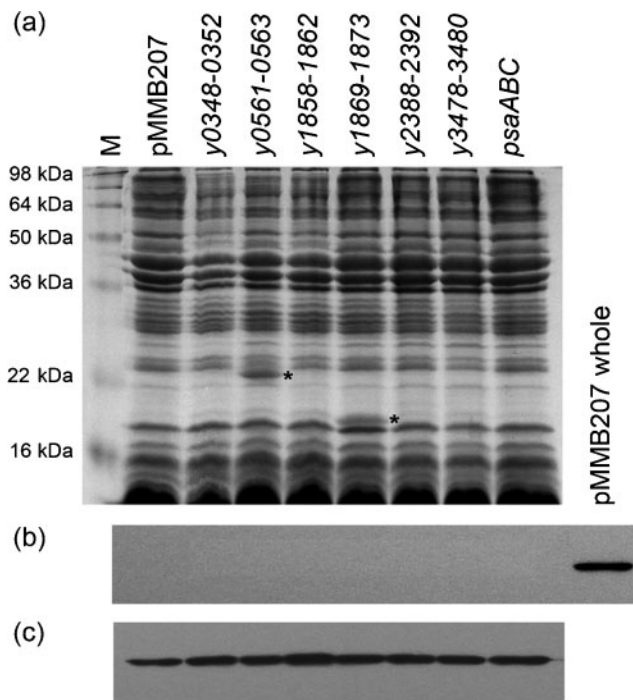


Fig. 2. Expression of chaperone/usher proteins in the outer membrane of *E. coli* AAEC185. Strains were cultured overnight under inducing conditions. Outer membranes were prepared and analysed by using Coomassie-blue-stained SDS-PAGE (a). Anti-RNA polymerase antibody demonstrated that the outer-membrane 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.

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

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. 3h). 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).

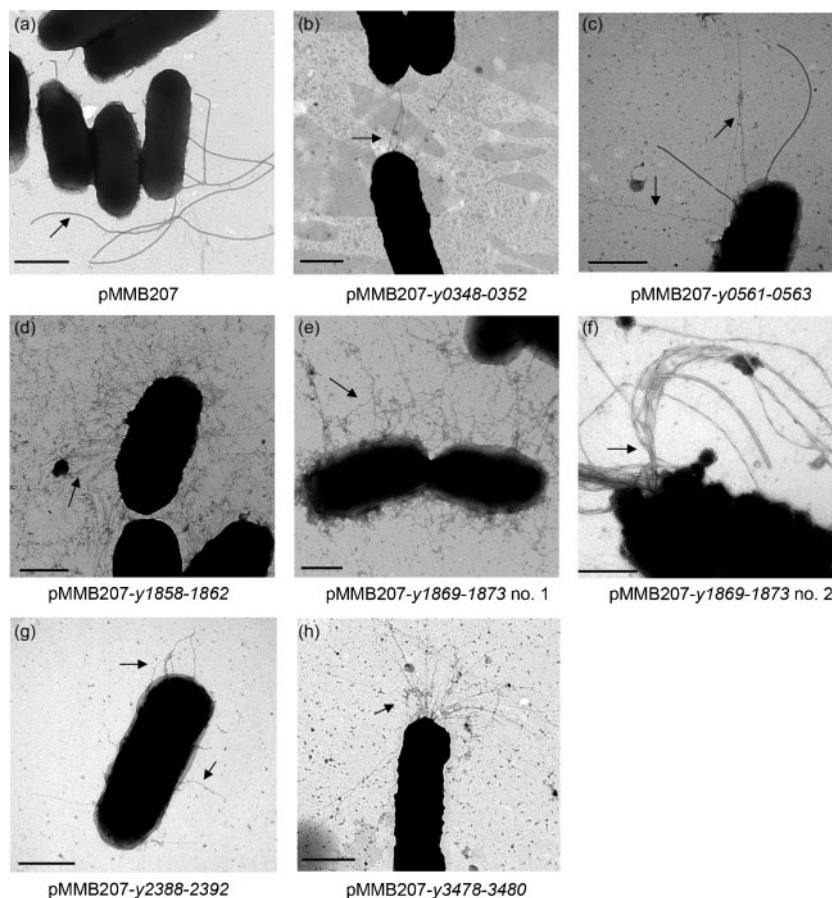


Fig. 3. EM images of *E. coli* AAEC185 (Δfim) expressing various *Y. pestis* chaperone/usher loci. Bacteria were induced for expression of the chaperone/usher loci at 37 °C with 50 µM IPTG. Arrows indicate structures of interest. In AAEC185 + pMMB207 (a), the arrow points to a flagellum; in the remaining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

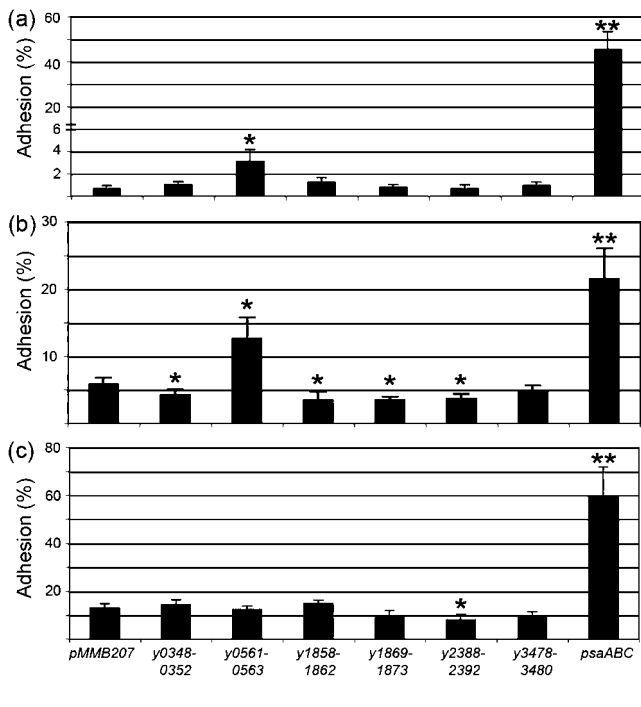


Fig. 4. Adhesion of *E. coli* AAEC185 expressing novel chaperone/usher systems or Psa to HEp-2 (a), THP-1 (b) and RAW264.7 macrophage (c) cells. Strains were cultured overnight at 28 °C on LB agar plates supplemented with 10 µg chloramphenicol ml⁻¹ and 100 µM IPTG. Bacteria were added to tissue culture 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 ± SD of three independent experiments with duplicate assays from each experiment. **P* < 0.05; ***P* < 0.0005.

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).

Adhesion of *Y. pestis* to cultured cells

Y. pestis and related pathogenic *Yersinia* species are known to produce a number of adhesins on their surface (Bliska *et al.*, 1993; El Tahir & Skurnik, 2001; Felek & Krukoni, 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 (Δ *psaA*) 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

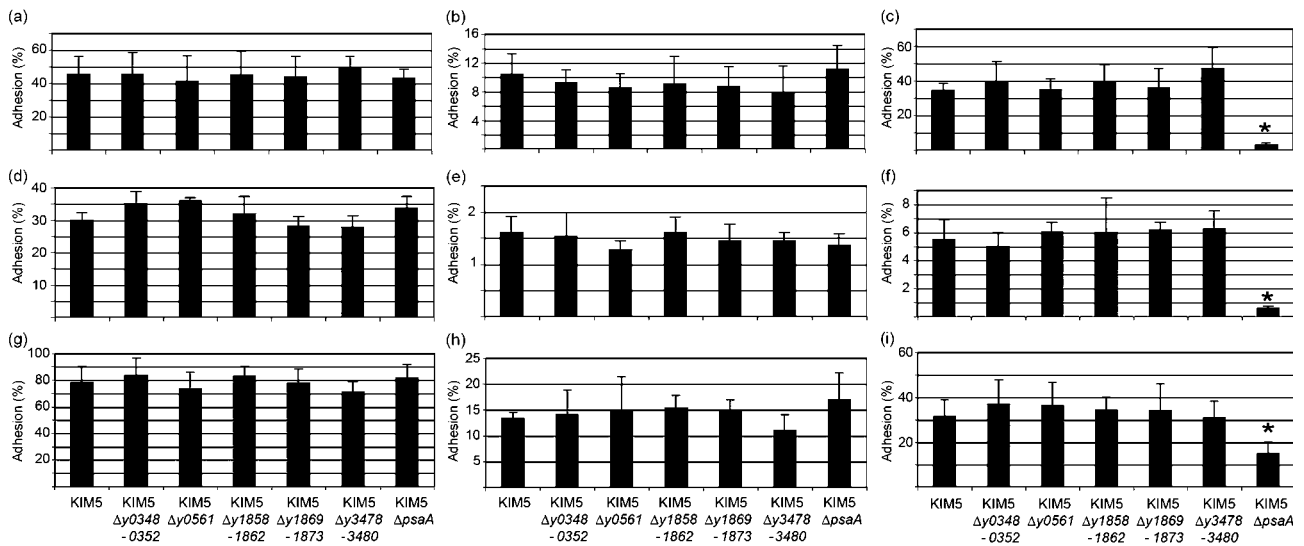


Fig. 5. Adhesion of *Y. pestis* chaperone/usher mutants to tissue culture cells. *Y. pestis* strains were added to HEp-2 (a–c), THP-1 (d–f) and RAW264.7 (g–i) cells at an m.o.i. of about 10. Cells were cultured at pH 7 28 °C (a, d, g), pH 7 37 °C (b, e, h) or pH 6 37 °C (c, f, i). THP-1 and RAW264.7 cells were blocked for phagocytosis by addition of 5 μg cytochalasin-D ml^{-1} . Results are means \pm SD of duplicates of three experiments on different days. * $P \leq 0.0003$.

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

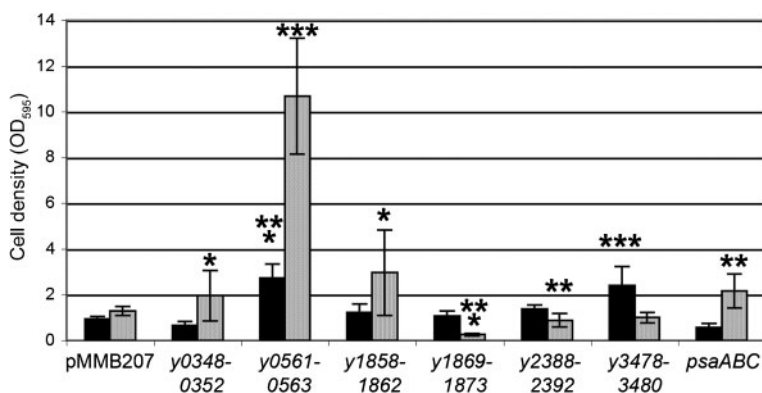


Fig. 6. Formation of biofilm-like deposits in *E. coli*. Overnight cultures of AAEC185 derivatives were diluted 1:100 into LB medium supplemented with 10 μg chloramphenicol ml^{-1} and 100 μM IPTG in polystyrene culture plates and incubated for 24 h at 28 °C (grey bars) or 37 °C (black bars). Plates were washed three times and attached bacteria were stained with 0.01% crystal violet. After three washes, crystal violet stain was solubilized with an 80% ethanol/20% acetone mixture and optical density was recorded at 595 nm and normalized for bacterial growth (Methods). Results are the means \pm SD of three independent experiments with at least duplicate assays from each experiment. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.00005$.

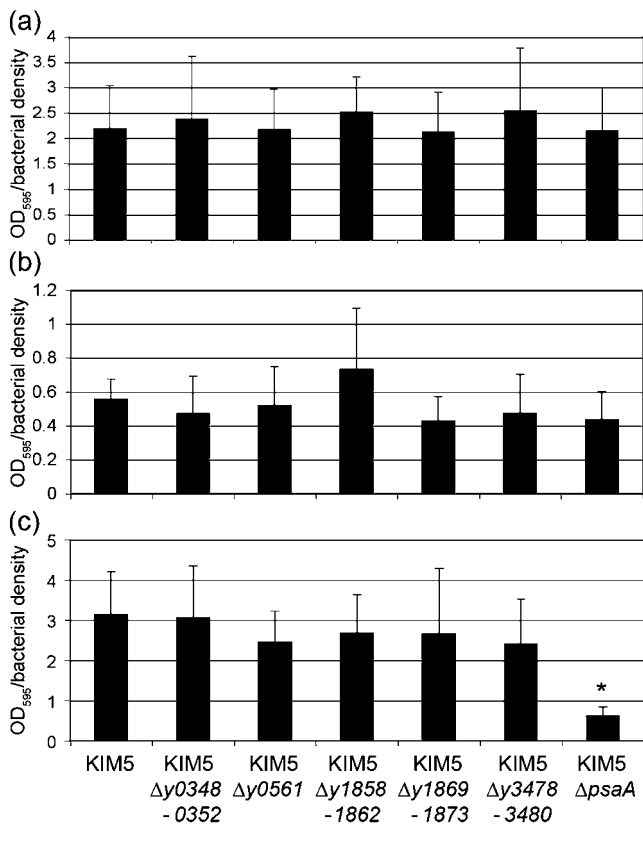


Fig. 7. The ability of *Y. pestis* chaperone/usher mutants to form *hms*-independent biofilms. KIM5 derivatives were grown in PMH2 minimal medium (Gong *et al.*, 2001) at 28 °C pH 7 (a), 37 °C pH 7 (b) or 37 °C pH 6 (c) and processed as described for *E. coli*. For *Y. pestis*, raw data for OD₅₉₅/bacterial density are presented without normalization. **P*=0.00001.

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 °C pH 7, HIB medium at 37 °C pH 7 and HIB medium at 37 °C pH 6. Cultures were grown overnight and expression levels were compared with the housekeeping gene *rpoB* (*rpoB*=1.0). Under each of these three growth conditions, none of the novel chaperone/usher systems was expressed well (all ≤ 0.02 relative to *rpoB*; Fig. 8 and Supplementary Table S4). Positive controls in this experiment were *cafI*, encoding the chaperone/usher-dependent F1 protein capsule of *Y. pestis* (known to be expressed at 37 °C), and *psaA*, known to be induced at 37 °C pH <6.7 (Ben-Efraim *et al.*, 1961). Both controls were induced appropriately in HIB, and the *16S rRNA* gene was strongly expressed at each condition. As a metabolic gene of intermediate expression, we included *gapA* (encoding glyceraldehyde 3-phosphate dehydrogenase A), which has been used previously in other qRT-PCR analyses

as a normalization control (Alteri & Mobley, 2007). *gapA* was expressed to slightly higher levels than *rpoB* under all three standard *in vitro* conditions.

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 °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 *cafI* and *psaA* loci. Under microaerophilic conditions at 37 °C, *cafI* was only modestly expressed, at levels 3.9-fold above *rpoB*, compared with expression that was 405-fold above *rpoB* when shaken at 37 °C pH 7 (Fig. 8 and Supplementary Table S4). Thus, oxygen tension strongly affects *cafI* 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 °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, *cafI* was not very strongly expressed in PMH3 at 37 °C (Fig. 8 and Supplementary Table S4), a temperature known to induce *cafI* 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 °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 °C pH 7.5 culture was only mildly acidic at pH 6.6 at the end of the experiment, while the 37 °C pH 7.5 and 37 °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 °C and pH <6.7 or a component of the minimal medium PMH3 induces expression of the *psaABC* locus, even at 28 °C. In any event, this result clearly indicates that *psaA* can be

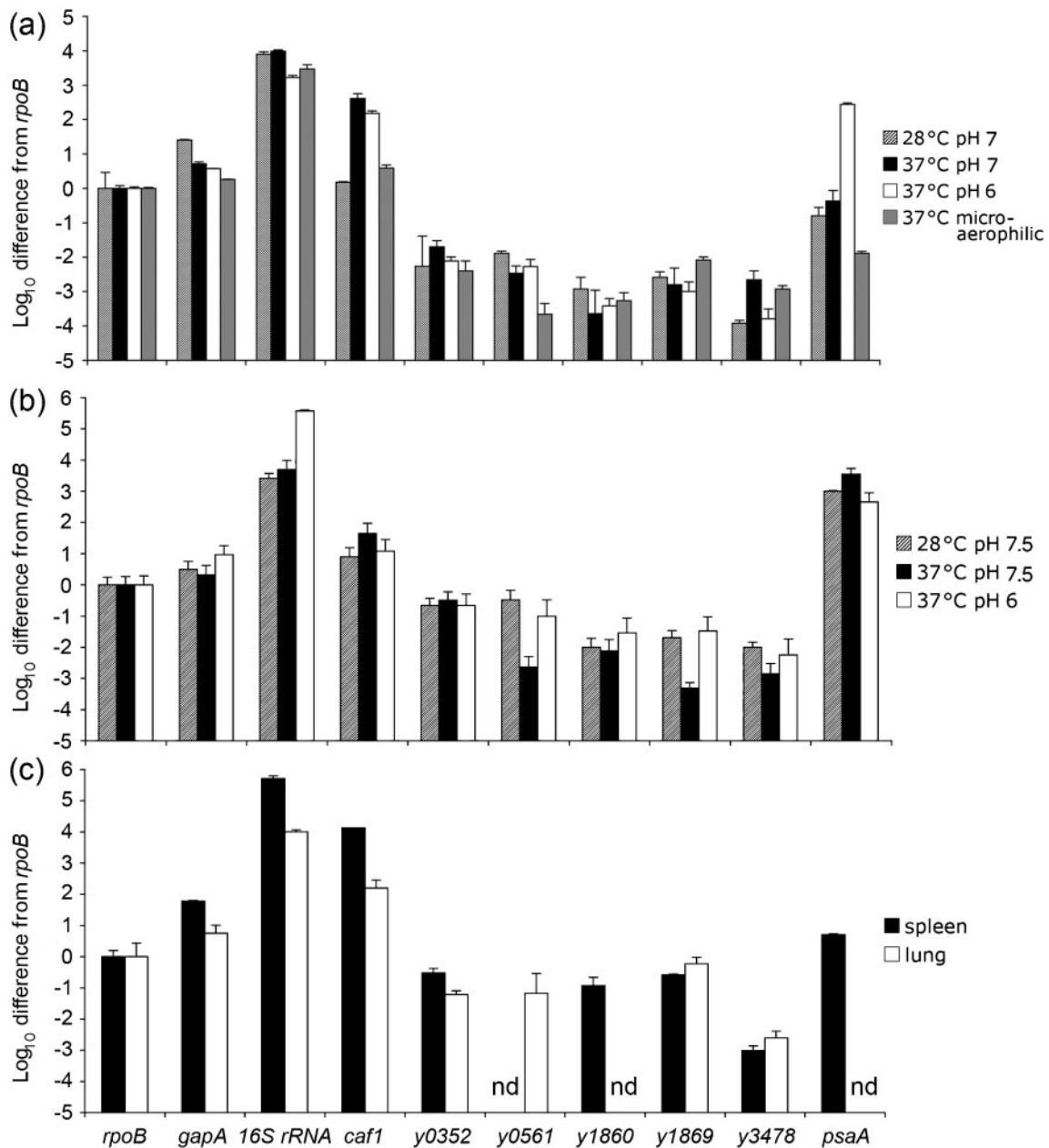


Fig. 8. qRT-PCR analysis of chaperone/usher system expression *in vitro* and *in vivo*. KIM5 was grown in HIB (a), PMH-3 (b) or harvested from infected tissue 3 days post-infection (c). Expression was normalized to *rpoB* and plotted on a log scale after subtracting the signal obtained from no reverse 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.

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 LD₅₀ (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

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, *cafI* 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 [\sim 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, $\Delta y1858-1862$, 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 $\Delta psaA$ 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 $\Delta psaA$ 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.

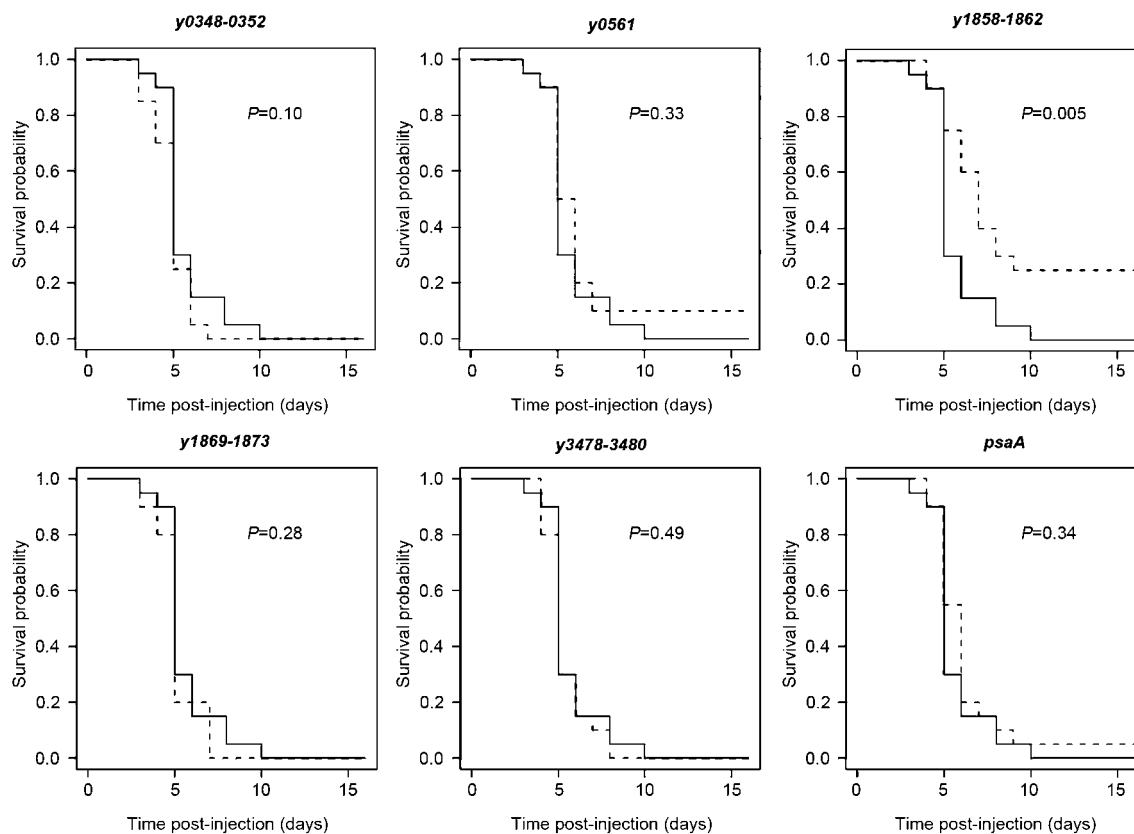


Fig. 9. Mouse survival assay for *Y. pestis* KIM5 and individual deletion mutants. Overnight cultures of *Y. pestis* KIM5, KIM5 pCD1⁻ and deletion mutants were intravenously inoculated into mice at a dose of 5–10 LD₅₀ (\sim 50 bacteria). Mice were observed for 14 days after injection for survival. Ten 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, $\gamma0561$ -0563 conferred adhesive activity to *E. coli* (Fig. 4), although deletion of $\gamma0561$ had no effect on *Y. pestis* cell attachment (Fig. 5). Given that *Y. pestis* has several other adhesins including Ail (Felek & Krukoniš, 2009; Kolodziejek *et al.*, 2007), plasminogen activator (Pla; Kienle *et al.*, 1992; Lahteenmaki *et al.*, 1998) and Psa (Felek *et al.*, 2008; Galvan *et al.*, 2007; Lindler *et al.*, 1990; Yang & Isberg, 1997), we assume they (and other potential adhesins) may mask any binding defect of the $\gamma0561$ 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, $\gamma1858$ -1862 and $\gamma1869$ -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 $\gamma0561$ -0563 locus confers both adhesive and biofilm-forming activities to *E. coli*, we considered the possibility that strains expressing $\gamma0561$ -0563 are non-specifically sticky to all surfaces. However, we found that *E. coli* expressing $\gamma0561$ -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 β 1-linked galactosyl residues in glycosphingolipids (Payne *et al.*, 1998) and to phosphatidylcholine (Galvan *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 Δ *psaA* 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 – $\gamma0352$ (spleen and minimal medium), $\gamma0561$ (minimal medium), $\gamma1860$ (spleen) and $\gamma1869$ (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 (*cafI*) and Psa (*psaA*) production were well expressed under conditions shown previously to induce their expression, 37 °C and pH 6, respectively. During our extensive analysis we observed some conditions where *cafI* would be predicted to be expressed to high levels (various 37 °C conditions) but was not. These included poor expression of *cafI* 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 fimbriae on the surface of the bacterium. All of these activities may contribute to the role of this locus in plague pathogenesis.

ACKNOWLEDGEMENTS

We thank Dr James Bliska for supplying *Y. pseudotuberculosis* strain IP2666, as a source of the *y2388-2392* chaperone/usher locus. We thank Susan Van Horn and the Central Microscopy Imaging Center (Stony Brook University) for assistance with EM. We thank Drs Creg Darby and Alexandra Koumoutsis for performing the *C. elegans* and Congo red experiments. Animal experiments were performed at the University of Michigan School of Dentistry in accordance with the standards of the AALAC-accredited Unit for Laboratory Animal Medicine (ULAM). This work was supported by grants from the University of Michigan Biomedical Research Council (BMRC), Office of the Vice President for Research (OVPR) and the Rackham Graduate School to E. S. K. and National Institutes of Health grant AI055621 to D. G. T.

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Edited by: V. Sperandio