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Phenotypic characterization of OmpX, an Ail homologue of *Yersinia pestis* KIM

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The goal of this study was to characterize the *Yersinia pestis* KIM OmpX protein. *Yersinia* spp. provide a model for studying several virulence processes including attachment to, and internalization by, host cells. For *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*, Ail, YadA and Inv, have been implicated in these processes. In *Y. pestis,* YadA and Inv are inactivated. Genomic analysis of two *Y. pestis* strains revealed four loci with sequence homology to Ail. One of these genes, designated y1324 in the *Y. pestis* KIM database, encodes a protein designated OmpX. The mature protein has a predicted molecular mass of 17.47 kDa, shares approximately 70 % sequence identity with *Y. enterocolitica* Ail, and has an identical homologue, designated Ail, in the *Y. pestis* CO92 database. The present study compared the *Y. pestis* KIM6$^+$ parental strain with a mutant derivative having an engineered disruption of the OmpX structural gene. The parental strain (and a merodiploid control strain) expressed OmpX at 28 and 37 °C, and the protein was detectable throughout all phases of growth. OmpX was required for efficient adherence to, and internalization by, cultured HEp-2 cell monolayers and conferred resistance to the bactericidal effect of human serum. Deletion of *ompX* resulted in a significantly reduced autoaggregation phenotype and loss of pellicle formation *in vitro*. These results suggest that *Y. pestis* OmpX shares functional homology with *Y. enterocolitica* Ail in adherence, internalization into epithelial cells and serum resistance.

INTRODUCTION

Yersinia pestis, the agent of plague, probably evolved from *Yersinia pseudotuberculosis* relatively recently, resulting in three pandemics (Wren, 2003). Today, treatment and prevention are not yet 100 % effective and a better understanding of pathogenesis is needed to enhance protection (McEvedy, 1988; Titball & Williamson, 2004; Zietz & Dunkelberg, 2004). *Y. pestis* carries a number of pathogenesis genes on the chromosome, but the pCD1 plasmid is essential for virulence. pCD1 encodes a type III secretion system and effector proteins with several functions, including circumventing host innate immunity (Leigh *et al.*, 2005; Lindler *et al.*, 1990).

Y. pestis enters epithelial cells, another mechanism to circumvent the immune response (Cowan *et al.*, 2000). In airborne disease, penetration of epithelial cells may promote development of pulmonary lesions (Liu *et al.*, 2006). Although Pla protease enhances interaction with epithelial cells (Cowan *et al.*, 2000; Lahteenmaki *et al.*, 1998, 2001a), Psa fimbria have also been implicated. Neither is sufficient to confer complete internalization. Thus, *Y. pestis* adherence and internalization mechanisms are not yet completely elucidated (Liu *et al.*, 2006).

Outer-membrane proteins (Omps) have β-strand structures with membrane-spanning domains, and participate in channelling, antibiotic resistance and signal transduction (Bockmann & Caflisch, 2005). One Omp, OmpX, was first described for *Enterobacter cloacae* (Stoorvogel *et al.*, 1991), but homologues, including PagC, Lom, Rck and Ail (the attachment–invasion locus protein of *Yersinia* spp.), were identified in other Gram-negative bacteria (Dupont *et al.*, 2004; Heffernan *et al.*, 1992a; Mecsas *et al.*, 1995). Proteins in this family promote invasion, resistance to complement-mediated killing, survival in macrophages, and internalization in epithelial cells (Cirillo *et al.*, 1996).

In the *Y. pestis* KIM genomic database, four ORFs for OmpX or Ail variants have been identified: y1324, y1682, y2034 (Caspi *et al.*, 2006; Karp *et al.*, 2005) and y2446 (UniProtKB/TrEMBL database). The y1324 gene encodes a protein which has a predicted molecular mass of 21 569 Da (including its signal sequence), has high sequence identity (99 and 68.5 %) to Ail in *Y. pseudotuberculosis* and *Yersinia*

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Abbreviations: C_T, threshold cycle; HIS, heat-inactivated serum; normal human serum.

enterocolitica, respectively, and is designated OmpX. The other three genes, y1682, y2034 and y2446 encode predicted proteins with 37.5, 46.4 and 45.9 %, amino acid sequence identity, respectively, with *Y. enterocolitica* Ail.

Although *Y. enterocolitica* Ail has been extensively studied (Miller *et al.*, 1990, 2001), similar studies have not been reported for its *Y. pestis* homologues. Two previous studies showed that loss of an unidentified protein in the *Y. pestis* outer membrane affects autoaggregation (Podladchikova & Rykova, 2006) and adhesion to phagocytes (Kukleva *et al.*, 2000). The present study was conducted to characterize *Y. pestis* OmpX by engineering an OmpX deletion mutation in *Y. pestis* KIM6$^+$ and comparing the properties of this mutant strain to the parental and complemented strains.

METHODS

Media, strains, and plasmids. Bacteria were cultured in Luria–Bertani (LB) low salt medium (EMD). Antibiotics were used at the following concentrations: nalidixic acid (Nal), 50 μg ml^{-1}; chloramphenicol (Cm), 30 μg ml^{-1}; and kanamycin (Kn), 50 μg ml^{-1}. LB agar with 5 % sucrose and lacking NaCl was used to select for double-crossover recombinants, employing the *sacBR* locus. Congo red agar was used to confirm the pigmentation phenotype of the *Y. pestis* KIM6$^+$ Nal^r strain (Surgalla & Beesley, 1969).

Strains and plasmids used in the study are listed in Table 1. A spontaneous Nal^r mutant was obtained by plating *Y. pestis* KIM6$^+$ on LB containing Nal. The pMS20 suicide plasmid, with *sacBR* encoding levensucrase, and Cm^r, was used as described previously (Gay *et al.*, 1983). In some experiments, *Y. pestis* strains constitutively expressing green fluorescent protein (GFP) were used. These were constructed by electroporating pFVP25.1 (a gift from G. Mallo; *Caenorhabdidis* Genetics Center, Minneapolis, MN; http://www.cbs.umn.edu/CGC), which encodes Amp^r and GFP.

HEp-2 cells (ATCC CCL-23) (Dziewanowska *et al.*, 2000) were grown in 6 % CO_2 (37 °C) in growth medium (GM) [low glucose Dulbecco's modified Eagle's medium (Gibco) supplemented with 10 % (v/v) fetal bovine serum (FBS) (HighClone) and 1 % (v/v) penicillin/streptomycin solution (Gibco)].

Construction of a *Y. pestis* KIM6$^+$ Nal^r *ompX* deletion mutant. The *ompX* gene was amplified by PCR using primers complementary to the predicted promoter and terminator regions and containing engineered *Xba*I and *Eco*RV sites, respectively. The resulting 750 bp PCR product was cloned into pMS20 (Smith, 2000) and the construct (pMHZ1) was transformed into *Escherichia coli* CC118 *λpir*. A representative clone was digested with *Mfe*I and *Nde*I (sites in *ompX*), generating a 426 bp deletion. A gene conferring Kn^r (neomycin phosphotransferase; *npt*), with flanking FRT (flippase recognition target) sites, was amplified by PCR using pKD4 (Datsenko & Wanner, 2000) as a template and, and cloned into pMHZ1. The resulting construct, pMHZ2 carrying Kn^r, was transformed into *E. coli* S17-1 *λpir*. A transformant harbouring pMHZ2 was mated with *Y. pestis* KIM6$^+$ Nal^r as described previously (Smith, 2000) and counter-selected on LB agar with Nal and Cm. This merodiploid strain (*ompX*$^+$/*ompX*::*npt*) was generated by a homologous single-crossover recombination. It was maintained and served as (i) a single copy *ompX* complementation control and (ii) an isogenic precursor for selecting the *ompX*::*npt* disruption. The latter was isolated on LB agar containing sucrose to select for a second crossover event while maintaining selection for the *ompX*::*npt* disruption. Sucrose-resistant, Cm-sensitive, colonies were tested by PCR for the *ompX*::*npt* disruption (generation of a 1828 bp product). DNA sequencing was used to confirm all constructs.

PCR. Primers (Table 1) were purchased from Integrated DNA Technologies and reagents were from Invitrogen. Primers used to amplify Tn*5 npt* contained engineered *Mfe*I and *Nde*I restriction sites. In the reverse primer, a mutation was introduced to exclude the *Nde*I restriction site naturally present in the FRT region. Amplification of this region, including the FRT sequences, enables deletion of the antibiotic resistance gene using the flippase-bearing pCP20 plasmid (Datsenko & Wanner, 2000). PCR products were visualized on agarose gels and sizes were determined using the 1 kb Plus DNA ladder (Fisher Scientific).

Expression of *ompX* and the downstream (y1325) *hcaT* gene were measured by real-time PCR. Total RNA was extracted as described previously (Rebeil *et al.*, 2006). Reverse transcription with hexanucleotides (Roche), real-time PCR using the SYBR green I dye master mix and an ABI 7000 thermocycler (Perkin–Elmer Applied Biosystems), and data analysis were as described previously (Seo *et al.*, 2007). Relative quantities of mRNA were normalized to the amount of *proS* mRNA in the samples. RNA isolation for each strain was done in duplicate on two different days and real-time PCR was performed in triplicate for each RNA sample.

Culture growth measurements. Growth rate comparisons were done in LB broth (50 ml) at 28 and 37 °C on an orbital shaker (200 r.p.m.). A Beckman Coulter DU530 spectrophotometer (Beckman Instruments) was used to measure OD_{600} of the cultures hourly, with vigorous vortexing before each reading.

Internalization assays. *Y. pestis* cells from ~12 h (OD_{600} 0.55–0.75) cultures grown at 28 °C (200 r.p.m.) in LB medium were washed in PBS (0.01 M sodium phosphate, 0.8% Nacl, pH 7.2) and resuspended in internalization medium (IM; GM lacking FBS and antibiotics). Dilutions were made in PBS containing 0.1 % Triton X-100 and 0.2 % glycerol to determine cell numbers by plate counts. HEp-2 cells (1.5×10^5 per well) in GM were incubated in 24-well plates (6 % CO_2, 37 °C). After 42 h, the cell monolayers were washed three times with IM and 1 ml adjusted *Y. pestis* suspension was added to each well to produce co-cultures with m.o.i. values between 10 and 20. The plates were centrifuged (5 min, 200 ***g***, 18 °C) and incubated for 1 h as described above. Each well was washed three times with IM and the co-cultures were incubated for 1.5 h in IM with 500 μg gentamicin per well (Gibco) to kill extracellular *Y. pestis*. The wells were washed three times and trypsin-like enzyme (Tryp-Le Express, Gibco) was added and incubated for 7 min to detach the HEp-2 cells. Triton X-100 (0.025 %) was added to release intracellular bacteria and bacterial numbers were determined from plate counts. Initial experiments, using standard techniques (Shaffer & Goldin, 1974), showed that inactivating *ompX* did not significantly alter the gentamicin minimal bactericidal concentration (MBC; 3.9–7.8 μg ml^{-1}).

Cell-association assays. Procedures were conducted as described above except that, after 1 h incubation, the HEp-2/*Y. pestis* co-cultures were washed nine times to remove unbound bacteria and the gentamicin exposure step was omitted. In some experiments, microscopy was used to assess cell association of *Y. pestis* strains expressing GFP from plasmid pFVP25.1. HEp-2 cells were incubated for 48 h in a four-chamber coverglass (Nalge Nunc International) starting with an initial inoculum of 5.0×10^4 bacteria ml^{-1}. The assays were performed as described above except that after the ninth wash the cells were fixed with 3.7 % formaldehyde and stained with 16.5 nM phalloidin conjugated with Alexa Fluor 546 as described by the manufacturer (Invitrogen). Images of nine 0.7 μm-thick slices collected by LSM5 Pa laser scanning microscope (Carl Zeiss MicroImaging) with a Plan-Apochromat 63 × /1.4 Oil DIC lens were processed by Zeiss LSM Image Examiner version 3.2.0.70.

Table 1. Strains, plasmids and PCR primers

Strain or plasmid	Relevant genotype	Reference or source
Strains		
E. coli CC118 *λpir*	R^- M^+ *λpir*$^+$	de Lorenzo *et al.* (1990)
E. coli S17-1 *λpir*	Δ*recA* RP4 2-Tc::*M*u-Kn::Tn*7* *λpir*$^+$ *tra*$^+$; Tp^r Str^r	de Lorenzo *et al.* (1990)
Y. pestis KIM6$^+$	*pgm*$^+$ pYV$^-$ pMT1	S. C. Straley, U. Kentucky
Y. pestis KIM6$^-$	*pgm*$^-$ *hmsT*$^+$ pYV$^-$ pMT1	S. C. Straley, U. Kentucky
Y. pestis KIM6$^+$ Nal^r	*pgm*$^+$ pYV$^-$ pMT1 Nal^r from *Y. pestis* KIM6$^+$	This study
Y. pestis KIM6$^+$ Nal^r Δ*ompX*::*npt*	*pgm*$^+$ pYV$^-$ Nal^r Δ*ompX*::*npt*	This study
Y. pestis KIM6$^+$ Nal^r *ompX*$^+$/*ompX*::*npt*	*pgm*$^+$ pYV$^-$ Nal^r *ompX*$^+$ with integrated pMHZ2; merodiploid for *ompX*	This study
Y. pestis KIM6$^+$ Nal^r pFVP25.1	Continuously expresses GFP	This study
Y. pestis KIM6$^+$ Nal^r Δ*ompX*::*npt* pFVP25.1	Continuously expresses GFP	This study
Y. pestis KIM6$^+$ Nal^r *ompX*$^+$/*ompX*::*npt* pFVP25.1	Continuously expresses GFP	This study
Plasmids		
pKD4	Amp^r; containing Kn^r gene flanked by FRT sites	Datsenko & Wanner (2000)
pMS20	*mob*$^+$, *pir*-dependent *ori*R6K, *sacBR* Cm^R	Smith (2000)
pMHZ1	pMS20 containing y1324 *ompX* gene from *Y. pestis*	This study
pMHZ2	pMS20 containing Δ*ompX*::*npt* flanked by FRT sites	This study
pFVP25.1	Amp^r; encodes GFP	G. Mallo *Caenorhabditis* Genetics Center
PCR primers: application and characteristics	**Primer sequence (F, forward; R, reverse)**	
Amplification of y1324 *ompX* gene	5′-CGCGTCTAGATCATGTGTCAGATATTTG-3′ (F) 5′-GCGCGATATCTAGCCTACCCCTATTAA-3′ (R)	This study
Disruption of *ompX* by insertion of *npt* cassette	5′-ACACGCCAATTGTTGTGTAGGCTGGAG-3′ (F) 5′-TATGTGCATATGATGGGAATTAGCCATGGTCCTCAATATCC-3′ (R)	This study
Confirmation of the mutation: flank *ompX* 200 bp 5′ and 3′ from the predicted ORF	5′-GCGCGATATCAACATTATCTGGTCCG-3′ (F) 5′-GCGCGGATCCTATTCATTGGTGACTTG-3′ (R)	This study
Detection of *hmsT* gene: predicted product length 639 bp	5′-TGATTGCGGTTATAAGCTTGAGC-3′ (F) 5′-GGCCATAGTTGTCGTTATAGGCTT-3′ (R)	This study
Detection of *hmsT* gene: predicted product length 1134 bp	5′-GTCTACTGACAGCACGATATTATGCAG-3′ (F) 5′-ATTTCTCTTGGCCCGGTAGAG-3′(R)	This study
Real-time PCR; *ompX* gene	5′-CATTTGCTCAGACTCGCCG-3′ (F) 5′-AGATGTTTTACTTCTACTCTCTGATTGACC-3′(R)	This study
Real-time PCR; *hcaT* gene	5′-TTTACTCTCCCGCTTCCTTGG-3′(F) 5′-CCAAAGCATCCGTCAATGGT-3′(R)	This study
Real-time PCR; *proS* gene	5′-GTGGTCATAAGTTGGTTGCGC-3′(F) 5′-GCACCGAAATCGCTCATCA-3′ (R)	This study

Analysis of OmpX expression. To examine OmpX levels at various growth phases and temperatures, bacteria were grown for 12 h as described above, washed in PBS, and pelleted by centrifugation at 12 000 ***g*** at 4 °C for 5 min. Proteins were extracted with a buffer containing 8 M urea (Sigma), 2 % CHAPS (EMD Chemicals), 20 mM DTT (Bio-Rad), bromophenol blue, Protease Inhibitors (Amersham Biosciences), and 100 × Nuclease (Amersham Biosciences). Protein samples and the Benchmark protein ladder (Invitrogen) were resolved by SDS-PAGE (Laemmli, 1970) on 12.5 % polyacrylamide gels and visualized by Coomassie blue staining.

Tandem mass spectrometry (MS/MS) analysis. Protein bands observed in SDS-PAGE gels were excised manually, destained, and subjected to standard protease digestion procedures (Shevchenko *et al.*, 1996). Samples were processed in high-recovery tubes from Axygen. Proteins were digested overnight at 37 °C using trypsin (Worthington) and 0.1 % n-octyl β-glucoside (Fluka) (Katayama *et al.*, 2001). Peptides were recovered with extraction buffer containing 50 % acetonitrile and 5 % trifluoroacetic acid and the resulting sample was concentrated under vacuum and resuspended in 5 % acetonitrile/0.1 % formic acid.

MS/MS analysis was performed using a Waters Nanoacquity ultra performance liquid chromatography (UPLC) unit, as described previously (Lee *et al.*, 2006). Digested peptides (2 µl) were loaded onto a fused silica capillary column (Atlantis dC18, 3 µm, 75 µm × 100 mm) in tandem with a trap column (Symmetry C18, 5 µm, 180 µm × 20 mm; Waters). The peptide mixture was separated using a gradient of 0.1 % formic acid in water and 0.1 % formic acid in acetonitrile at 0.4 µl min^{-1}. The column effluent was delivered directly to a Premiere quadrupole-time-of-flight mass spectrometer (Waters) equipped with a nanospray electrospray ion source. The MS and UPLC were controlled and mass spectra were acquired and analysed using MassLynx 4.0 software. All spectra were obtained in the positive ion mode. The survey scans used a 400–2000 Da mass range in continuum mode and up to three peptides with charges 2, 3 or 4 were sequenced (MS/MS) at a given retention time using 50–2000 Da mass window.

ProteinLynx Global Server 2.2 and Protein Expression Informatics System software Version 1.0 were used for MS/MS spectra analysis, peptide sequencing and protein identification. MS/MS data were compared to protein sequence databases from the University of Wisconsin (http://www.genome.wisc.edu/sequencing/pestis.htm) and the Sanger Institute (http://www.sanger.ac.uk/Projects/Y_pestis/) and results were analysed using Mascot software (Matrix Science).

Serum-resistance assays. Venous blood was collected from healthy donors and processed as described previously (Vandenbosch *et al.*, 1987) to obtain normal human serum (NHS). Heat-inactivated serum (HIS) was generated at 56 °C for 30 min to inactivate complement. During the exponential phase of growth, bacterial cells (grown as above) were collected, washed twice in PBS, diluted 100-fold, mixed with an equal volume of NHS or HIS, and incubated at 37 °C. Viable bacteria were quantified by plate counts as described above following incubation in sera for 0, 0.5 or 1.0 h.

Statistical analysis. Data were analysed using either the Mann–Whitney or Student's *t* tests. These analyses were conducted with SigmaPlot 9.0 (Systat Software) software.

RESULTS

Bioinformatic analysis and generation of a *Y. pestis* KIM6$^+$ OmpX deletion mutant

Database analysis revealed two *Y. pestis* ORFs for OmpX or its homologue, Ail. UniProtKB/TrEMBL contains OmpX (*Y. pestis* KIM) beginning with GTG, and with a predicted 38 residue signal peptide (Fig. 1). Ail (Sanger Institute; *Y. pestis* CO92) begins with ATG and encodes a putative 26-residue signal peptide. ORF Finder (NCBI Tools for Bioinformatics Research) predicted the signal peptide sequence in the *Y. pestis* CO92 database for both genes.

Our strategy to investigate the role of OmpX in *Y. pestis* was to first construct a deletion mutation marked by a removable *npt* cassette encoding Kn^r. Primers designed to amplify *ompX* with flanking sequences yielded a predicted 750 bp PCR product (results not shown). Disruption of the amplified *ompX* required deletion of a 427 bp internal fragment, and replacing it with the *npt* gene and flanking sequences. This *ompX*::*npt* fragment was subcloned into a suicide plasmid (pMS20), generating pMHZ2 (Table 1). pMHZ2 was introduced into *Y. pestis* KIM6$^+$ Nal^r by conjugation. *Y. pestis* KIM6$^+$ Nal^r Cm^r colonies represented pMHZ2 co-integrates with an *ompX*/*ompX*::*npt* genotype as confirmed by PCR (results not shown). PCR analysis of one isolate generated the predicted 750 bp product representing chromosomal *ompX*, and the predicted 1829 bp product representing the introduced *ompX*::*npt* fragment. This *ompX* merodiploid strain (*Y. pestis ompX*$^+$/*ompX*::*npt*) was used as a positive control for subsequent experiments examining the properties of the *Y. pestis ompX::npt* strain. A second crossover, deleting the wild-type chromosomal *ompX* and the pMHZ2 co-integrate, and conferring Cm^r, was selected on LB agar with Kn and sucrose. A sucrose-resistant colony, that was also Kn^r and Cm^s, was chosen for further study after confirming the *ompX* disruption by PCR. PCR analysis of this *Y. pestis ompX::npt* strain generated only the predicted 1829 bp fragment (results not shown).

Real-time PCR data indicated that *ompX* transcription was not significantly different in the parental and complemented strains (mean change in threshold cycle (ΔC_T): −6.550 and −6.285; P=0.182), and expression by the *Y. pestis ompX::npt* deletion mutant was undetectable. Furthermore, expression of the downstream y1325 (*hcaT*) gene was not significantly different in any strain [mean ΔC_T:

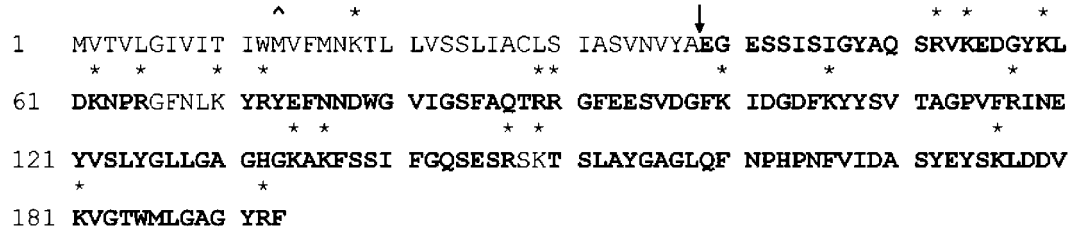

Fig. 1. Amino acid sequencing and bioinformatic analysis of potential OmpX translation products. Peptides detected by MS/MS analysis are indicated in bold. The arrow indicates the beginning of sequence coverage and the probable signal sequence cleavage site. Asterisks indicate sites potentially susceptible to trypsin. Met at position 1 represents the translation start site predicted in the UniProtKB/TrEMBL database for *Y. pestis* KIM. The Met marked by ^ is the predicted translation start site for Ail in the *Y. pestis* CO92 protein database from the Sanger Institute. The latter site was predicted for both strains using NCBI Tools for Bioinformatics Research software.

8.16 (parental), 8.82 (deletion), 8.22 (merodiploid); $P=0.279$ (parental vs deletion), and $P=0.907$ (parental vs merodiploid)].

Proteomic analysis

SDS-PAGE analysis of *Y. pestis* whole-cell lysates (Fig. 2) indicated that the *Y. pestis* KIM6$^+$ Nal^r Δ*ompX*::*npt* strain lacked a protein band at approx. 19 kDa which was present in parental *Y. pestis* KIM6$^+$ Nal^r, control merodiploid *Y. pestis* KIM6$^+$ Nal^r *ompX*$^+$/*ompX*::*npt*, and *Y. pestis* KIM6$^-$ strains. This protein was expressed at both 28 and 37 °C and during exponential and stationary phases. This protein band from gels containing the parental strain, and corresponding gel slices from the mutants, was excised, digested with trypsin, and sequenced. MS/MS data were used to search protein databases for *Y. pestis* KIM and *Y. pestis* CO92. This revealed that the missing protein was identical to the mature form of OmpX (gene tag y1324) and Ail (gene tag ypo2905) in these two databases, respectively. In this report, we used the nomenclature in the *Y. pestis* KIM database (OmpX rather than Ail) to be consistent with the gene designation used in the database for the strain most closely related to the strain used in our study.

Sequence coverage by ion mapping started at a putative signal peptide cleavage site predicted with SignalP 3.0 Server (Bendtsen *et al.*, 2004; Nielsen & Krogh, 1998) (Fig. 1). This suggests that the signal peptide is cleaved between Ala and Glu (Fig. 1); *Y. enterocolitica* Ail is cleaved at an analogous site (Miller *et al.*, 1990). The predicted molecular mass of mature OmpX, calculated with ProtParameters (Gasteiger *et al.*, 2005), is 17.47 kDa, slightly smaller than the ~19 kDa size estimated by SDS-PAGE.

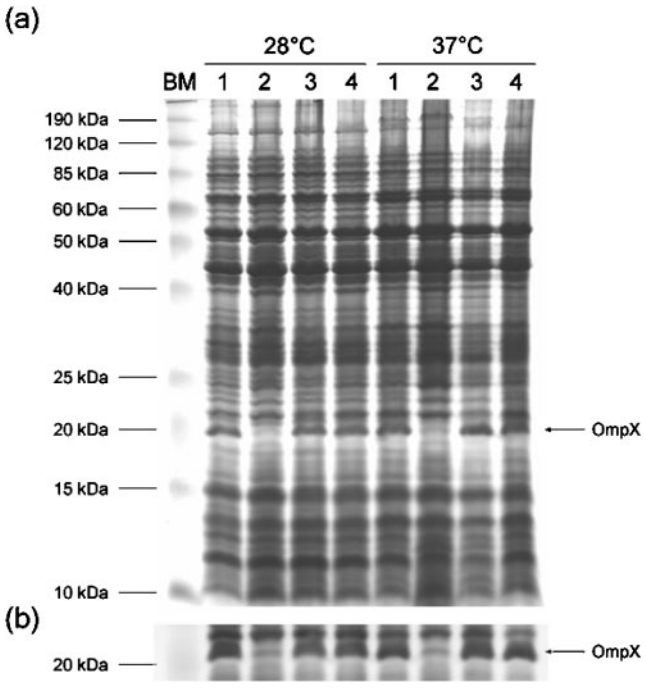

Fig. 2. SDS-PAGE of *Y. pestis* whole-cell lysate proteins. Cultures were grown at either 28 or 37 °C and collected during (a) mid-exponential phase (OD_{600} 0.7–0.8) or (b) near the start of the stationary (OD_{600} 2.1–2.3) phase of growth. (lanes 1, *Y. pestis* KIM6$^+$ Nal^r; lanes 2, *Y. pestis* KIM6$^+$ Nal^r Δ*ompX*::*npt*; lanes 3, *Y. pestis* KIM6$^+$ Nal^r, *ompX*$^+$/*ompX*::*npt*; lanes 4: *Y. pestis* KIM6$^-$). The lane labelled BM contains BenchMark pre-stained protein standard. The gel shows results from a representative experiment that was performed twice.

Disruption of *ompX* does not impair the growth of *Y. pestis in vitro*

To ascertain whether the mutation affected bacterial growth rate, growth kinetics were monitored for the parental strain and its mutant derivatives at 28 and 37 °C. The *ompX* disruption did not impair growth at either temperature. In fact, the *Y. pestis* KIM6$^+$ Nal^r Δ*ompX*::*npt* deletion mutant had a small but reproducible increase in growth rate at 28 °C compared to the parental and merodiploid control strains (doubling times 97, 101 and 107 min respectively; data not shown). This increased growth rate was more prominent at 37 °C; the deletion mutant had a doubling time of 118 min compared to 152 and 154 min, respectively, for the parental and merodiploid control strains. When compared to the parental and control strains, the increase in growth rate was statistically significant ($P<0.009$) for cultures grown at 37 °C, but not for those grown at 28 °C.

Disruption of *ompX* results in loss of autoaggregation and pellicle formation

Certain strains of *Y. pestis* produce characteristic flocculent growth in broth cultures (Bobrov *et al.*, 2002). This phenotype was readily observable for the parental *Y. pestis* KIM6$^+$ Nal^r strain, the merodiploid *Y. pestis* KIM6$^+$ Nal^r *ompX*$^+$/*ompX*::*npt* control strain, and the *pgm*-deficient strain *Y. pestis* KIM6$^-$ (Fig. 3a), which autoaggregated when grown at either 28 or 37 °C. Despite being subjected to shaking, the bacterial growth settled to the bottom when grown at 28 °C. Smaller aggregates formed but remained dispersed in cultures grown at 37 °C. In addition, these strains formed pellicles attaching to the sides of the tubes at the air–liquid interface. Loss of these characteristics coincided with disruption of the *ompX* gene. *Y. pestis* KIM6$^+$ Nal^r Δ*ompX*::*npt* grew as a homogeneous suspension at both 28 and 37 °C, and neither clumping nor pellicle formation was observed at either temperature. Microscopic analysis revealed that the parental and merodiploid strains formed large aggregates, while the *Y. pestis* KIM6$^+$ Nal^r Δ*ompX*::*npt* mutant grew as individual cells or in smaller clusters (Fig. 3b).

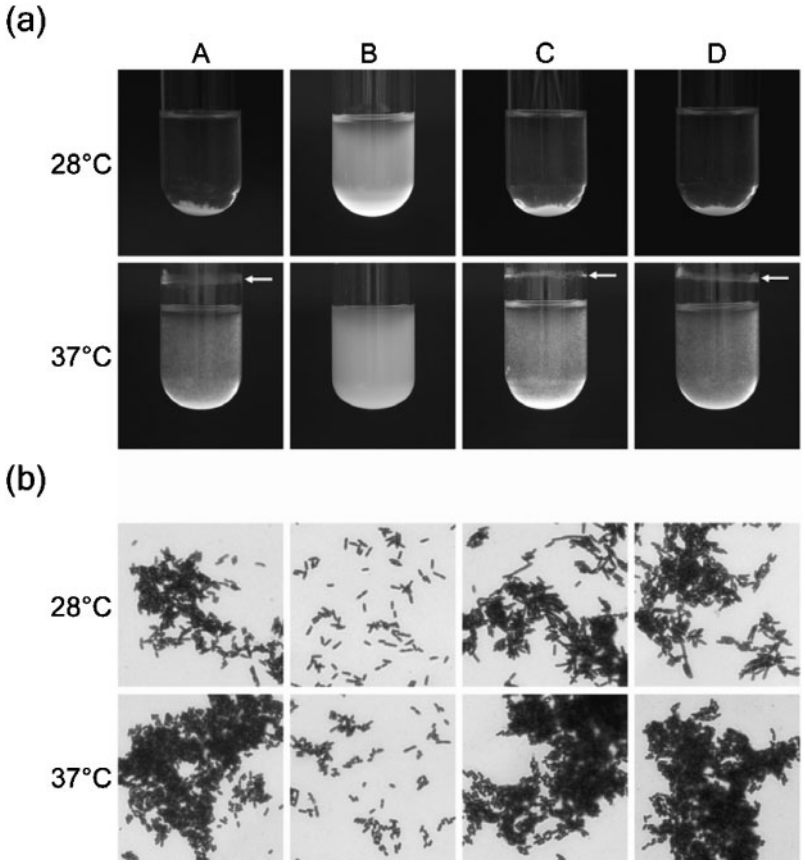

Fig. 3. Changes in autoaggregation and pellicle formation induced by disruption of the *ompX* gene. Bacteria were incubated in LB broth at 28 or 37 °C with vigorous shaking (200 r.p.m.) and collected during mid-exponential phase. (a) Macroscopic observation of cultures demonstrating autoaggregation and pellicle formation (indicated by arrows). A, *Y. pestis* KIM6$^+$ Nal^r; B, *Y. pestis* KIM6$^+$ Nal^r Δ*ompX*::*npt*; C, *Y. pestis* KIM6$^+$ Nal^r, *ompX*$^+$/*ompX*::*npt*; D, *Y. pestis* KIM6$^-$. Note: Pellicle formation at 28 °C is very light and not easily visible or photographed in cultures collected during exponential growth. (b) Microscopic observation (taken at ×600 magnification) of crystal-violet-stained cells confirming loss of autoaggregation upon deletion of the *ompX* gene.

OmpX protein and *Y. pestis* interactions with human epithelial cells

It was previously reported that two OmpX homologues, Ail and Rck in *Y. enterocolitica* and *Salmonella typhimurium*, respectively, are involved in adherence to and internalization by epithelial cells (Cirillo *et al.*, 1996; Heffernan *et al.*, 1994; Miller & Falkow, 1988). To determine whether *Y. pestis* OmpX performs similar functions, we examined the effect of inactivating the *ompX* gene on interactions with cultured HEp-2 human epithelial cells. The overall association of *Y. pestis* KIM6$^+$ Nal^r Δ*ompX*::*npt* with HEp-2 cells was significantly impaired in comparison to *Y. pestis* KIM6$^+$ Nal^r and *Y. pestis* KIM6$^+$ Nal^r *ompX*$^+$/*ompX*::*npt* (Fig. 4). Microscopic observation of *Y. pestis* KIM6$^+$ Nal^r Δ*ompX*::*npt*-HEp-2 co-cultures revealed that very few bacterial cells were associated with the HEp-2 cells. In contrast, the parental and merodiploid control strains of *Y. pestis*, both of which express OmpX, were observed predominantly as large aggregates associated with Hep-2 cell monolayers. Bacterial cell counts from the cell-association assays were consistent with microscopic observation. *Y. pestis* KIM6$^+$ Nal^r Δ*ompX*::*npt* cell numbers associated with HEp-2 cells (representing both adherent and internalized cells) were reduced by ~90 % (11-fold reduction) compared to the parental strain and merodiploid control. This led to an ~98 % (65.5-fold) reduction in internalization of *Y. pestis*. The data indicated that internalization was more severely affected than adherence. Specifically, only 6 % of the cell-associated *Y. pestis* KIM6$^+$ Nal^r Δ*ompX*::*npt* were internalized by eukaryotic cells vs 35.75 and 32 % for *Y. pestis* KIM6$^+$ Nal^r and *Y. pestis* KIM6$^+$ Nal^r *ompX*$^+$/*ompX*::*npt*, respectively.

OmpX confers resistance to human serum

To examine whether *Y. pestis* OmpX confers resistance to killing by complement in human serum, a property of *Y. enterocolitica* and *Y. pseudotuberculosis* Ail (Bliska & Falkow, 1992; Yang *et al.*, 1996), standard assay protocols were used. The *ompX* deletion mutant was significantly more susceptible to complement-mediated killing compared to the parental and control strains (Fig. 5) grown at both 28 and 37 °C. Incubation in 50 % NHS at 37 °C was essentially 100 % lethal to *Y. pestis* KIM6$^+$ Nal^r *ompX*::*npt* after 1 h. In contrast, *Y. pestis* KIM6$^+$ Nal^r and *Y. pestis* KIM6$^+$ Nal^r *ompX*$^+$/*ompX*::*npt* expressing OmpX both retained complete viability during this time period. To exclude the possibility that the autoaggregation phenotype of the parental strain may significantly distort the complement-resistance results by protecting a fraction of the bacterial population from complement by making the bacterial cells inaccessible, we observed that during longer incubation with serum, bacterial aggregates were being

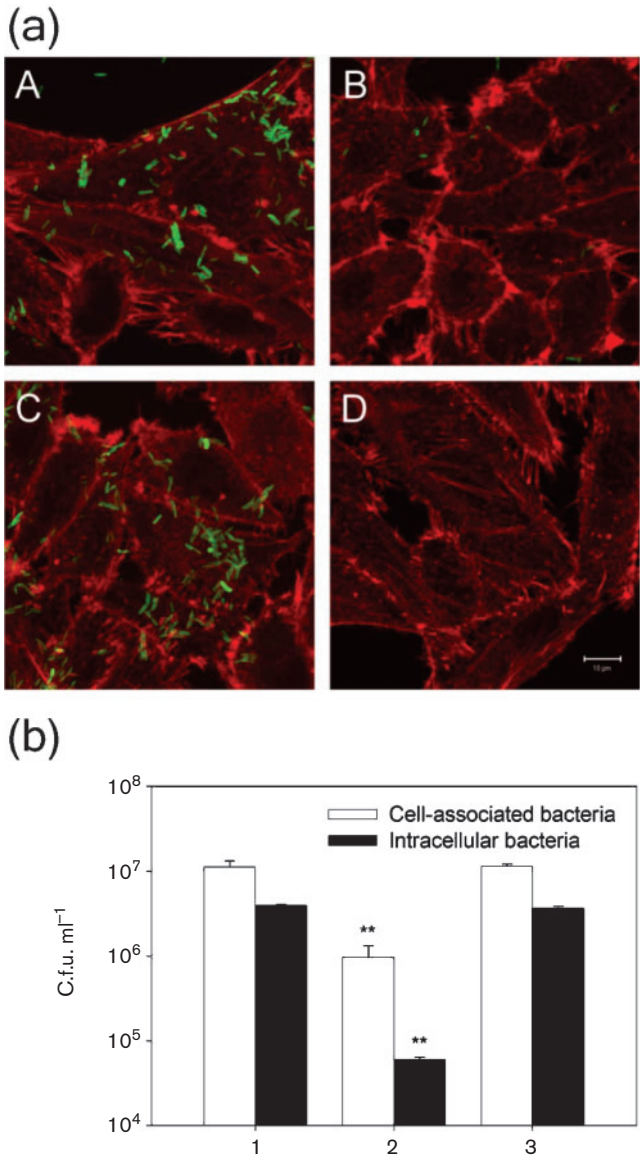

Fig. 4. The OmpX protein in *Y. pestis* KIM6$^+$ Nal^r is required for optimal interaction with HEp-2 cells. (a) Cell-associated GFP-expressing *Y. pestis* bacteria after 1 h incubation and exhaustive washing. *Y. pestis* KIM6$^+$ Nal^r Δ*ompX*::*npt* pFVP25.1 (B) is visible as mostly individual cells associated with HEp-2 cells. In contrast, *Y. pestis* KIM6$^+$ Nal^r pFVP25.1 (A) and *Y. pestis* KIM6$^+$ Nal^r *ompX*$^+$/*ompX*::*npt* pFVP25.1 (C) associated with the HEp-2 cells exist in bacterial aggregates and more bacteria are cell-associated compared to the *ompX* deletion strain. D, HEp-2 cell monolayers incubated without bacteria (taken at ×634 magnification; bar, 10 μm). (b) Quantification of cell association and internalization by: (1) *Y. pestis* KIM6$^+$ Nal^r, (2) *Y. pestis* KIM6$^+$ Nal^r Δ*ompX*::*npt* and (3) *Y. pestis* KIM6$^+$ Nal^r *ompX*$^+$/*ompX*::*npt*. Results shown are mean ± SEM from data derived from two assays performed in duplicate (n=8) on two separate days. The asterisks (**) indicate statistically significant differences between the deletion mutant, and the parental and control strains (P<0.001).

disrupted and after 4 h the cells were more separated (data obtained from confocal microscopy of GFP-labelled bacteria; not shown). Incubation of the bacteria with serum for 4 h and subsequent addition of fresh serum did not result in any decrease in viability, which indicates that it is OmpX, not autoaggregation, which is the major factor in serum resistance.

DISCUSSION

This study characterized *Y. pestis* KIM6$^+$ OmpX by inactivating the *ompX* gene and assessing the effects of the mutation on phenotypes associated with homologues in other Gram-negative organisms. We determined that *ompX* endows *Y. pestis* with the abilities to (i) efficiently associate with and become internalized by epithelial cells, (ii) resist the bactericidal activity of human serum, and (iii) promote autoaggregation.

In conducting this study we searched the *Y. pestis* KIM and CO92 databases and encountered conflicting nomenclature; the protein in the KIM6 database was designated OmpX, whereas the homologue in the CO92 strain was designated Ail. Although an Ail designation could be appropriate, because our work was conducted with *Y. pestis* KIM6$^+$, we used the OmpX designation in this report to be consistent with the current database designation. Furthermore, *Y. pestis* has three additional *ail*-like genes with varying levels of predicted sequence similarity, and to designate *ompX* as *ail* would require a further qualifier as to which *ail* homologue was being addressed. Until a unified nomenclature is assigned, we have used *ompX* to limit potential confusion. However, we recommend that a standard nomenclature be assigned through future research and discussions.

Inactivation of *ompX* reduced epithelial cell association and internalization ~90 and ~98 %, respectively. At present we cannot distinguish whether the increased cell association of *Y. pestis* expressing OmpX is due directly to the effects of OmpX or indirectly due to OmpX-mediated autoaggregation. The residual cell association observed for the *ompX* deletion mutant is consistent with evidence suggesting that *Y. pestis* interaction with non-professional phagocytes is multifactorial. Kukleva *et al.* (2000) noted that a 22 kDa protein in the *Y. pestis* outer membrane promoted adherence to phagocytes; the protein in their study was not identified, nor were its effects on non-professional phagocytes analysed. Other proteins are involved in adhesion and invasion by *Y. pestis* (Cowan *et al.*, 2000; Lahteenmaki *et al.*, 1998, 2001a, b, 2003; Leigh *et al.*, 2005; Liu *et al.*, 2006). The role of the pCP1 plasmid, encoding the plasminogen activator (Pla protease), in interaction with HeLa cells has been reported (Cowan *et al.*, 2000). Although the reduced cell association and internalization resulting from pCP1 curing was less than that caused by inactivating *ompX* in *Y. pestis* KIM6$^+$, one cannot make direct comparisons since different cell culture systems and

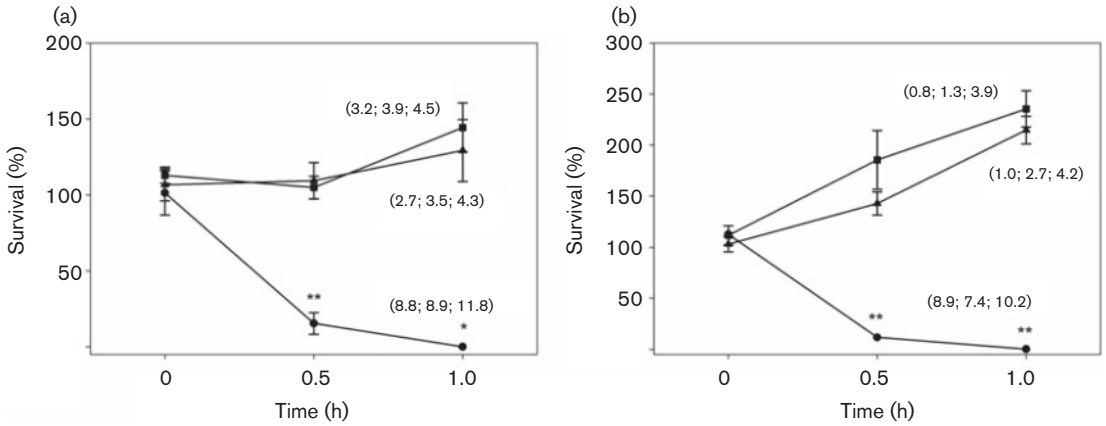

Fig. 5. OmpX promotes resistance to human serum bactericidal activity when the bacteria are grown at either 28°C (a) or 37 °C (b). Bacteria were incubated in 50 % NHS or HIS. ▲, *Y. pestis* KIM6$^+$ Nal^r; ●, *Y. pestis* KIM6$^+$ Nal^r Δ*ompX*::*npt*; ■, *Y. pestis* KIM6$^+$ Nal^r *ompX*$^+$/*ompX*::*npt*. The number of surviving bacteria after incubation with NHS is presented as a percentage of the number of bacteria incubated in HIS (100 %). Data in parentheses represent bacterial cell numbers for each strain (×10^5) following incubation in HIS for 0, 0.5, and 1 h respectively. These values represent 100 % survival in HIS at each time point. Results are the mean ± SEM from data derived from two assays performed on separate days (n=6). * and ** indicate statistically significant differences (P<0.002 and P<0.001, respectively) at the 0.5 and 1 h time points between the deletion mutant and either of the other two strains tested.

strains were used. Psa fimbriae are also adhesins, but have not been reported to promote internalization (Liu *et al.*, 2006).

Y. pestis OmpX, in contrast to *Y. enterocolitica* Ail, mediates adherence and internalization when bacteria are grown at <30 °C. We propose two potential explanations for this difference. The first is that *Y. enterocolitic*a Ail expression is temperature-regulated; low levels of Ail are produced at 28 °C and much higher levels are produced at 37 °C (Bliska & Falkow, 1992; Pierson & Falkow, 1993). However, *Y. pestis* OmpX expression is constitutive. The second is that artificial overexpression of *Y. enterocolitica* Ail at 28 °C does not promote internalization of *Y. enterocolitica,* indicating that additional factors contribute to Ail-mediated internalization (Bliska & Falkow, 1992). As shown previously, adherence conferred by *Y. enterocolitica* Ail is affected by the length or structure of O side-chains in lipopolysaccharide (LPS) which are temperature-regulated (Pierson, 1994). Presumably because of steric interference, only bacteria with shortened O side-chains (*Y. enterocolitica* grown at 37 °C) can promote interaction between Ail and the host cell (Bliska & Falkow, 1992; Pierson, 1994). In contrast to *Y. enterocolitica, Y. pestis* LPS, due to mutation, lacks O-antigen regardless of growth temperature (Prior *et al.*, 2001). This lack of O-antigen interference could explain OmpX-mediated internalization at low temperature. The length of O-antigen has been previously shown to regulate functions of other proteins such as Pla and PgtE (Kukkonen *et al.*, 2004). LPS structural diversity (Skurnik *et al.*, 2000; Zhou *et al.*, 2004) might also explain differences between OmpX in *Y. pestis* and Ail in *Y. pseudotuberculosis*. Despite sharing nearly 100 % sequence homology with *Y. pestis* OmpX, Ail fails to confer adherence of *Y. pseudotuberculosis* to HEp-2 cells (Yang *et al.*, 1996). Another possible explanation of this discrepancy is the one substitution (Phe vs Val) in the predicted third (out of four) surface-exposed-loop regions of *Y. pseudotuberculosis* Ail. Resolution of this issue by complementing the *ompX*::*npt* mutant with *ail* is a goal of future studies.

Like *Y. enterocolitica* Ail and *S. typhimurium* Rck, OmpX mediates serum resistance (Heffernan *et al.*, 1994; Miller *et al.*, 1989, 1990). Disruption of *ompX* in this study increased sensitivity of *Y. pestis* cultured at either 28 or 37 °C. Resistance was attributed to a direct protective effect by OmpX from complement-mediated killing, rather than shielding of the bacteria from serum components within cell aggregates. Incubating the parental strain in the presence of serum caused complete dissociation of the cell aggregates by 4 h; yet the cells remained resistant to fresh serum added to the suspension at this point (results not shown). The protection by OmpX at either 28 or 37 °C contrasts with *Y. enterocolitica* Ail, which confers this trait only at 37 °C (Bliska & Falkow, 1992; Pierson & Falkow, 1993; Pierson, 1994), reflecting temperature regulation of Ail expression in that organism (Bliska & Falkow, 1992). OmpX expression at both temperatures probably helps ensure *Y. pestis* complement resistance by organisms multiplying in humans or following growth at ambient temperatures. It is clear that OmpX plays a significant role in serum resistance. Since the strains used in this study are wild-type with respect to Pla protease, our results are consistent with a previous report which demonstrated that deletion of Pla protease, known to degrade C3, does not

apparently increase sensitivity to serum (Sodeinde *et al.*, 1992). Comparative studies of another member of the Ail family, Rck of *S. typhimurium*, may suggest that the mode of OmpX action is through inhibition of C9 polymerization and formation of the mature membrane attack complex (MAC) on the bacterial surface (Heffernan *et al.*, 1992b). It has also been reported that *Y. pestis* binds the complement regulatory protein C4bp as observed by Ngampasutadol *et al.* (2005). Whether *Y. pestis* OmpX is directly involved in these latter two processes is currently being examined.

The autoaggregation phenotype associated with pellicle formation and flocculent growth is characteristic of certain *Y. pestis* strains and connected with virulence (Laird & Cavanaugh, 1980). The parental KIM6$^+$ strain has this property, which was lost by *Y. pestis* KIM6$^+$ Δ*ompX*::*npt* grown at either 28 or 37 °C. These phenotypes have been associated with biofilms in some bacteria such as *Campylobacter jejuni* (Joshua *et al.*, 2006), *Mycobacterium smegmatis* (Chen *et al.*, 2006), and *Pseudomonas aeruginosa* (Friedman & Kolter, 2004) and are attributed to resistance to host defence mechanisms (Schembri *et al.*, 2003). Other *Y. pestis* genetic loci, including *hmsHFRS* (in the *pgm* locus) and *hmsT* (Hare & McDonough, 1999), have been correlated with autoaggregation. The presence of *hmsHFRS* and *hmsT* in the *Y. pestis* strains used in this study was confirmed by growth on Congo red agar and by PCR, respectively (data not shown), excluding the possibility that the altered autoaggregation phenotype was attributed to loss of either *hmsHFRS* or *hmsT*. We also demonstrated that *Y. pestis* KIM6$^-$, lacking *pgm*, autoaggregated. This finding supports studies by Podladchikova & Rykova (2006) on derivatives of the *Y. pestis* EV76 *hms*$^-$ pYV strain in which an unidentified bacterial cell surface protein of ~17 kDa was involved in autoaggregation.

In summary, we have demonstrated a prominent role for *Y. pestis* OmpX in several potential virulence processes. Of three genes (*inv*, *yadA* and *ail*) associated with these processes in enteropathogenic *Yersinia*, *yadA* (Rosqvist *et al.*, 1988) and *inv* (Simonet *et al.*, 1996) are inactive in *Y. pestis*. Experiments are ongoing to investigate other roles of OmpX in pathogenesis and its potential application as a vaccine candidate.

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