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 KIM 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
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 & others, 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+ Nalr strain (Surgalla & Beesley, 1969).

Strains and plasmids used in the study are listed in Table 1. A spontaneous Nalr mutant was obtained by plating Y. pestis KIM6+ on LB containing Nal. The pMS20 suicide plasmid, with sacBR encoding leuvenscensure and Cm, 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; Caenorhabditis Genetics Center, Minneapolis, MN; http://www.cbs.umn.edu/CGC), which encodes Ampr and GFP.

HEp-2 cells (ATCC CCL-23) (Dziwanowska et al., 2000) were grown in 6 % CO2 (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+ Nalr ompX deletion mutant.

The ompX gene was amplified by PCR using primers complementary to the predicted promoter and terminator regions and containing engineered Xhol and EcoRV sites, respectively. The resulting 750 bp PCR product was cloned into pMS20 (Smith, 2000) and the construct (pMHZ1) was transformed into Escherichia coli CC118/p pir. A representative clone was digested with MfeI and Ndel (sites in ompX), generating a 426 bp deletion. A gene conferring Knr (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 Knr, was transformed into E. coli S17-1/p pir. A transformant harbouirng pMHZ2 was mated with Y. pestis KIM6+ Nalr as described previously (Smith, 2000) and counter-selected on LB agar with Nal and Cm. This merodiploid strain (ompX::npt) was generated by a homologous single-cross-over 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 Tns npt contained engineered MfeI and NdeI restriction sites. In the reverse primer, a mutation was introduced to exclude the NdeI 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 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 OD600 of the cultures hourly, with vigorous vortexing before each reading.

#### Internalization assays

Y. pestis cells from ~2 h (OD600 0.5–0.7) 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 × 105 per well) in GM were incubated in 24-well plates (6 % CO2, 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 × 106 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 0.7 μm-thick slices collected by LSM5 Pa laser scanning microscope (Carl Zeiss MicroImaging) with a Plan-Apochromat 63 × 1.4 Oil DIC lenses were processed by Zeiss LSM Image Examiner version 3.2.0.70.
Table 1. Strains, plasmids and PCR primers

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype</th>
<th>Reference or source</th>
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<tr>
<td><strong>Strains</strong></td>
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<tr>
<td>E. coli CC118 Δpir</td>
<td>R− M+ Δpir+</td>
<td>de Lorenzo et al. (1990)</td>
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<tr>
<td>E. coli S17-1 Δpir</td>
<td>ΔrecA RP4 2-Tc::Mu-Kn::Tn7 Δpir+ tra+; Tp' Str'</td>
<td>de Lorenzo et al. (1990)</td>
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<td>Y. pestis KIM6+</td>
<td>pgm+ pYV− pMT1</td>
<td>S. C. Straley, U. Kentucky</td>
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<tr>
<td>Y. pestis KIM6−</td>
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<tr>
<td>Y. pestis KIM6+ Na'</td>
<td>pgm− pYV− pMT1 Na' from Y. pestis KIM6+</td>
<td>This study</td>
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<td>Y. pestis KIM6+ Na'</td>
<td>pgm+ pYV− Na' ΔompX::npt</td>
<td>This study</td>
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<td>ΔompX::npt</td>
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<td>Y. pestis KIM6+ Na' ompX+ / ompX::npt</td>
<td>pgm+ pYV− Na' ompX+ with integrated pMHZ2; merodiploid for ompX</td>
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<td><strong>Plasmids</strong></td>
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<td>pKD4</td>
<td>AmpR; containing Kn' gene flanked by FRT sites</td>
<td>Datsenko &amp; Wanner (2000)</td>
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<td>pMHZ1</td>
<td>pMS20 containing y1324 ompX gene from Y. pestis</td>
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<td>pMHZ2</td>
<td>pMS20 containing ΔompX::npt flanked by FRT sites</td>
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<td>pFVP25.1</td>
<td>AmpR; encodes GFP</td>
<td>G. Mallo Caenorhabditis Genetics Center</td>
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<td><strong>PCR primers: application and characteristics</strong></td>
<td><strong>Primer sequence (F, forward; R, reverse)</strong></td>
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<td>Amplification of y1324 ompX gene</td>
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<td>Confirmation of the mutation: flank ompX 200 bp 5' and 3' from the predicted ORF</td>
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<td>Real-time PCR; ompX gene</td>
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<td>Real-time PCR; proS gene</td>
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<td></td>
<td>5'-GCACCGAATCGCTCATCA-3' (R)</td>
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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 12000 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 Axogen. 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.
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'. 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+ by conjugation. Y. pestis KIM6+ Kn' Cm' colonies represented pMHZ2 co-integrates with an 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::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', was selected on LB agar with Kn and sucrose. A sucrose-resistant colony, that was also Kn' and Cm', 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 (∆Ct): -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 ∆Ct:

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**RESULTS**

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**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.
Proteomic analysis

SDS-PAGE analysis of Y. pestis whole-cell lysates (Fig. 2) indicated that the Y. pestis KIM6⁺ Nal⁻ ΔompX::npt strain lacked a protein band at approx. 19 kDa which was present in parental Y. pestis KIM6⁺ Nal⁺, control merodiploid Y. pestis KIM6⁺ Nal⁺ 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.

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⁻ ΔompX::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⁻ strain, the merodiploid Y. pestis KIM6⁺ Nal⁻ 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⁻ ΔompX::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⁻ ΔompX::npt mutant grew as individual cells or in smaller clusters (Fig. 3b).
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⁺ompX::npt with HEp-2 cells was significantly impaired in comparison to *Y. pestis* KIM6⁺ Nal⁺ and *Y. pestis* KIM6⁺ Nal⁺ ompX⁺/ompX::npt (Fig. 4). Microscopic observation of *Y. pestis* KIM6⁺ Nal⁺ ∆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⁺ ∆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⁺ ∆ompX::npt were internalized by eukaryotic cells vs 35.75 and 32% for *Y. pestis* KIM6⁺ Nal⁺ and *Y. pestis* KIM6⁺ Nal⁺ 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⁺ ∆ompX::npt after 1 h. In contrast, *Y. pestis* KIM6⁺ Nal⁺ and *Y. pestis* KIM6⁺ Nal⁺ 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...
Properties of \textit{Y. pestis} OmpX

DISCUSSION

This study characterized \textit{Y. pestis} KIM6\(^+\) OmpX by inactivating the \textit{ompX} gene and assessing the effects of the mutation on phenotypes associated with homologues in other Gram-negative organisms. We determined that \textit{ompX} endows \textit{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 \textit{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 \textit{Y. pestis} KIM6\(^+\), we used the OmpX designation in this report to be consistent with the current database designation. Furthermore, \textit{Y. pestis} has three additional \textit{ail}-like genes with varying levels of predicted sequence similarity, and to designate \textit{ompX} as \textit{ail} would require a further qualifier as to which \textit{ail} homologue was being addressed. Until a unified nomenclature is assigned, we have used \textit{ompX} to limit potential confusion. However, we recommend that a standard nomenclature be assigned through future research and discussions.

Inactivation of \textit{ompX} reduced epithelial cell association and internalization ~90 and ~98\%, respectively. At present we cannot distinguish whether the increased cell association of \textit{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 \textit{ompX} deletion mutant is consistent with evidence suggesting that \textit{Y. pestis} interaction with non-professional phagocytes is multifactorial. Kukleva \textit{et al.} (2000) noted that a 22 kDa protein in the \textit{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 \textit{Y. pestis} (Cowan \textit{et al.}, 2000; Lahteenmaki \textit{et al.}, 1998, 2001a, b, 2003; Leigh \textit{et al.}, 2005; Liu \textit{et al.}, 2006). The role of the pCP1 plasmid, encoding the plasminogen activator (Pla protease), in interaction with HeLa cells has been reported (Cowan \textit{et al.}, 2000). Although the reduced cell association and internalization resulting from pCP1 curing was less than that caused by inactivating \textit{ompX} in \textit{Y. pestis} KIM6\(^+\), one cannot make direct comparisons since different cell culture systems and 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.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig4.png}
\caption{The OmpX protein in \textit{Y. pestis} KIM6\(^+\) Na\(^+\) is required for optimal interaction with HEp-2 cells. (a) Cell-associated GFP-expressing \textit{Y. pestis} bacteria after 1 h incubation and exhaustive washing. \textit{Y. pestis} KIM6\(^+\) Na\(^+\) \textit{ompX}\text{}\textunderscore pFVP25.1 (B) is visible as mostly individual cells associated with HEp-2 cells. In contrast, \textit{Y. pestis} KIM6\(^-\) Na\(^+\) pFVP25.1 (A) and \textit{Y. pestis} KIM6\(^+\) Na\(^+\) \textit{ompX}\text{}\textunderscore pFVP25.1 (C) associated with the HEp-2 cells exist in bacterial aggregates and more bacteria are cell-associated compared to the \textit{ompX} deletion strain. D, HEp-2 cell monolayers incubated without bacteria (taken at \(×634\) magnification; bar, 10 \(\mu\)m). (b) Quantification of cell association and internalization by: (1) \textit{Y. pestis} KIM6\(^+\) Na\(^+\), (2) \textit{Y. pestis} KIM6\(^+\) Na\(^+\) \textit{ompX}\text{}\textunderscore pFVP25.1 and (3) \textit{Y. pestis} KIM6\(^+\) Na\(^+\) \textit{ompX}/\textit{ompX}\text{}\textunderscore pFVP25.1. 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 (\(P<0.001\)).}
\end{figure}
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. enterocolitica 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

![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+ Naft; ●, Y. pestis KIM6+ Naf ΔompX::npt; ■, Y. pestis KIM6+ Naft 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 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.](image-url)
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+ ΔompX::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 Podlachikova & 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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