Published in final edited form as:
Microb Pathog. 2012 January ; 52(1): 41–46. doi:10.1016/j.micpath.2011.09.009.

The Outer Membrane Protein A (OmpA) of *Y. pestis* promotes intracellular survival and virulence in mice

Sara Schesser Bartra[a], **Xin Gong**[b,*], **Cherish D. Lorica**[c], **Chaitanya Jain**[d], **Manoj K. M. Nair**[e], **Dieter Schifferli**[e], **Lianfen Qian**[f], **Zhongwei Li**[b], **Gregory V. Plano**[a], and **Kurt Schesser**[a]

[a]Department of Microbiology and Immunology, University of Miami Miller School of Medicine, 1600 NW 10th Ave, Miami, FL 33136, USA

[b]Department of Biomedical Science, Charles E. Schmidt College of Medicine, Florida Atlantic University, Boca Raton, FL 33431, USA

[c]Department of Pediatrics, University of Miami Miller School of Medicine, 1600 NW 10th Ave, Miami, FL 33136, USA

[d]Department of Biochemistry and Molecular Biology, University of Miami Miller School of Medicine, 1600 NW 10th Ave, Miami, FL 33136, USA

[e]Department of Pathobiology, University of Pennsylvania School of Veterinary Medicine, 3800 Spruce Street, Philadelphia, PA 19104, USA

[f]Department of Mathematics, Charles E. Schmidt College of Science, Florida Atlantic University, Boca Raton, FL 33431, USA

Abstract

The plague bacterium *Yersinia pestis* has a number of well-described strategies to protect itself from both host cells and soluble factors. In an effort to identify additional anti-host factors, we employed a transposon site hybridization (TraSH)-based approach to screen 10^5 *Y. pestis* mutants in an *in vitro* infection system. In addition to loci encoding various components of the well-characterized type III secretion system (T3SS), our screen unambiguously identified *ompA* as a pro-survival gene. We go on to show that an engineered *Y. pestis* Δ*ompA* strain, as well as a Δ*ompA* strain of the closely related pathogen *Y. pseudotuberculosis*, have fully functioning T3SSs but are specifically defective in surviving within macrophages. Additionally, the *Y. pestis* Δ*ompA* strain was outcompeted by the wild-type strain in a mouse co-infection assay. Unlike in other bacterial pathogens in which OmpA can promote adherence, invasion, or serum resistance, the OmpA of *Y. pestis* is restricted to enhancing intracellular survival. Our data show that OmpA of the pathogenic *Yersinia* is a virulence factor on par with the T3SS.

Keywords

Pathogenesis; Virulence; *Yersinia pestis*; OmpA; intracellular

Corresponding author: Tel.: +1-305-243-4760; fax: +1-305-243-4623; kschesser@med.miami.edu (K. Schesser).
*Current address: Department of Ophthalmology, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA

1. Introduction

Gram-negative bacteria possess an outer membrane (OM) that is composed of LPS, phospholipids, and proteins. The best characterized of the latter is the *E. coli* OmpA which has become a popular model for studies on membrane protein structure and folding dynamics (Smith *et al.* 2007). A variety of studies have also shown that in pathogenic *E. coli* and *Klebsiella pneumonia* OmpA mediates a wide-range of activities including resistance to complement and antibacterial peptides, and invasion of and survival within eukaryotic cells (Weiser and Gotschlich 1991; Prasadarao *et al.* 1996; Fu *et al.* 2003; Llobet *et al.* 2009).

The plague bacterium *Yersinia pestis* expresses a number of virulence factors that enhances its survival and proliferation within the mammalian host including a type III secretion (T3S) system for protection against immune cells and the outer membrane protein Ail which confers protection against complement-mediated killing (Viboud and Bliska 2005; Bartra *et al.* 2008). Several loci have been shown to be critical for the intracellular survival of *Y. pestis* including *ripA*, *mgtC*, and *ugd* which encode putative acetyl CoA transferase, Mg^{2+} acquisition, and an intermediary product involved in promoting resistance to antimicrobial peptides, respectively (Pujol *et al.* 2005; Grabenstein *et al.* 2006). It is becoming increasingly clear that although *Y. pestis* possesses a potent 'anti-phagocytosis' activity (mediated by the T3SS), it has also evolved robust intracellular survival mechanisms as either a failsafe or alternative virulence strategy.

To identify *Y. pestis* genes that impact its interaction with macrophages, we screened a highly complex pool of *Y. pestis* transposon mutants in a cell culture infection model. This infection model has been previously shown to be sensitive to defects in the T3S system (Bartra *et al.* 2001). Here we report that, in addition to T3S-associated genes (as predicted from single mutant studies), our efforts revealed that OmpA is specifically required for the intracellular survival of *Y. pestis* and the related pathogen *Y. pseudotuberculosis* in macrophages.

2. Results and Discussion

2.1. Identifying OmpA as a pro-survival factor in a cell culture infection model

We used a transposon site hybridization (TraSH)-based approach (Sassetti *et al.* 2003) to identify *Y. pestis* genes that play a pro-survival function during infection. A culture containing approximately 90,000 unique *Y. pestis* transposon mutants were generated and used to infect cultured mouse macrophage-like RAW 264.7 cells (Fig.1). After a 14-hr infection period, the surviving cell-associated bacteria were collected and used for a second round of infection following which bacterial DNA was isolated and used to create probes that were applied to tiled microarrays. Mutants that survived and/or proliferated during the infection will generate positive signals in the corresponding spots of the array whereas those mutants that were eliminated during the infection will fail to show signals thus possibly indicating that the matching gene is important for survival during infection. DNA was isolated from a parallel culture of the library of *Y. pestis* mutants that was propagated in the absence of macrophages in order to control for genes important for normal growth. More details of the *Y. pestis* mutagenesis, infection, and data analysis are described in *Methods* and Fig. 1.

Using a high statistical cut-off, we identified 44 open reading frames (ORFs) that were specifically important for optimal infection. A number of these ORFs (17) are located on the extrachromosomal plasmids pCD1 and pPCP1 that encode the components of the T3SS and the pla protease, respectively, both of which have been shown to play central role in *Y. pestis* virulence (Viboud and Bliska 2005). Among the remaining genes, we were particularly

interested that the ORF encoding the outer membrane protein A (*ompA*; y2735) was ranked among the various T3SS-encoding loci in terms of statistical significance (Fig.2A). The OmpA-encoding locus was the only representative among the numerous outer membrane proteins of *Y. pestis* that played a pro-survival function in the infection assay (Fig.2B).

To confirm that OmpA is specifically required for *Y. pestis* to optimally infect macrophages, an *ompA* deletion strain was constructed as well as strains possessing deletions in *ompC* (y2966), *ompF* (y2759), *ompN* (y2983) and the previously described *ail* (y1324) (Bartra *et al.* 2008). These strains were individually tested in an infection assay similar to that used in the TraSH-based screen except that the 'outputs' (the surviving bacterial fraction) was assessed by viable plating. The infectivity of these mutant strains were compared to that of the parental *Y. pestis* KIM5 strain and to an isogenic strain lacking YopB, a T3SS translocon component. Similar to that described previously (Rosenzweig *et al.*, 2005), there was an approximately 50-fold increase in the number of cell-associated wild-type *Y. pestis* during an 8-hour infection period whereas the number of cell-associated Δ*yopB* bacteria remained essentially unchanged during the infection period (Fig. 3A). The *Y. pestis* Δ*ompA* strain exhibited an intermediate level of infectivity compared to the wild-type and Δ*yopB* strains (Fig. 3A) in contrast to the strains deleted for the *ompC*, *ompF*, *ompN* or *ail* genes which resembled the wild-type parental strain in this assay (*data not shown*). The proliferation of the *Y. pestis* wild-type, Δ*yopB*, and Δ*ompA* strains in wells without macrophages was comparable indicating that under these conditions there is not a general growth defect (Fig. 3A). The expression of OmpA *in trans* in the Δ*ompA* strain increased the infectivity compared to the Δ*ompA* strain transformed with the vector control (Fig. 3A, *inset*). These data confirm the results of the TraSH-based screen indicating that OmpA promotes the survival of *Y. pestis* in the presence of macrophages.

Previously we have shown that the macrophage infection assay used above is sensitive to defects in either the T3SS itself (e.g., YopB) or factors that affect T3SS functioning (e.g., RNase E) (Bartra *et al.* 2001; Yang *et al.* 2008). We therefore employed two different assays to determine whether the lack of OmpA in *Y. pestis* impacted T3SS activity. In a secretion assay in the absence of macrophages, the wild-type, Δ*yopB*, and Δ*ompA* strains secreted the T3SS effector YopE in a similar manner (Fig. 3B). We also tested whether OmpA was required for the efficient delivery of Yop effectors into eukaryotic cells in an infection assay. Cells infected with the wild-type *Y. pestis* strain display a rounded morphology ('cytotoxicity') that is primarily due to the translocation of the YopE effector into the cytosol (Rosqvist *et al.* 1990). There were no detectable differences between the levels of cytotoxicity induced by the wild-type and Δ*ompA* strains in contrast to a strain lacking Ail, an adhesion required for optimal Yop delivery (Fig. 3C; Tsang *et al.* 2010). These results indicate that the *Y. pestis* Δ*ompA* strain possesses a functional T3SS that is able to efficiently secrete and translocate Yop proteins into eukaryotic cells. Consequently, the diminished growth of Δ*ompA* strains in the presence of macrophages does not appear to be due to defects in T3SS function.

2.2. OmpA promotes the intracellular survival of Y. pestis and Y. pseudotuberculosis

Previously it has been shown that *Yersinia* spp. including *Y. pestis*, are able to survive within macrophages (Finegold 1969; Une 1977; Tabrizi & Robins-Browne 1992; Yamamoto *et al.* 1996; Oyston *et al.* 2000). This characteristic of the yersiniae does not involve the T3SS (Zhang *et al.*, 2008). We therefore determined whether the reduced infectivity of the Δ*ompA* strain observed above could be, at least in part, accounted for by a reduction in intracellular survival. To promote the internalization of the *Y. pestis ompA*$^{+}$ and Δ*ompA* strains we used strains lacking the T3SS-encoding pCD1 virulence plasmid (ΔpCD1). The initial uptake of the *Y. pestis* ΔpCD1 Δ*ompA* and the isogenic *ompA*$^{+}$ strain into cultured macrophages were comparable; however, upon a longer infection period the survival of the Δ*ompA* strain was

significantly reduced compared to that of the *ompA*$^+$ strain (Fig. 4). These data show that OmpA promotes the intracellular survival of *Y. pestis.*

The OmpA protein expressed by the enteropathogenic *Y. pseudotuberculosis* is identical to the OmpA of *Y. pestis*. We therefore generated a *Y. pseudotuberculosis ΔompA* deletion strain to investigate whether OmpA promoted the intracellular survival of *Y. pseudotuberculosis*. For these infection assays, we used the *Y. pseudotuberculosis* YPIII strain harboring the T3SS-encoding virulence plasmid pIB102 (Bolin *et al.* 1982). The initial uptake of the wild-type *Y. pseudotuberculosis* strain is much lower than that of the *ΔyopB* strain (Fig. 5); the enhanced uptake of the *ΔyopB* strain is characteristic of T3SS-defective strains and is due the anti-phagocytic activities of the Yop effectors (Hakansson *et al.*, 1996). However, despite the differences in their uptake, the wild-type and Δ*yopB* strains displayed comparable levels of intracellular survival (Fig. 5) consistent with the studies of *Y. pestis* cited above showing that intracellular survival is independent of the T3SS. Even though the uptake of the *Y. pseudotuberculosis* Δ*ompA* strain resembled that of the wild-type strain (which would be expected if the activities of their respective T3SSs were comparable), the intracellular survival of this strain is clearly reduced compared to the wild-type and Δ*yopB* strains (Fig. 5). These data indicate that OmpA in *Y. pseudotuberculosis*, like OmpA of *Y. pestis*, enhances intracellular survival within macrophages.

2.3. Comparable analysis of OmpA regulation and function in Y. pestis and E. coli

For a variety of reasons OmpA has been extensively studied in *E. coli*. For example, in *E. coli* it has been shown that OmpA confers resistance to serum factors (Weiser and Gotschlich 1991). However, we could not detect any differences between the *Y. pestis* wild-type and *ΔompA* strains using a serum killing assay which we have previously employed in our characterization of Ail (*data not shown*; Bartra *et al.* 2008). OmpA is a highly abundant outer membrane protein in *E. coli* that is preferentially expressed in growing cells and is readily visualized by Coomassie staining outer membrane preparations fractionated by SDS-PAGE (Smith *et al.* 2007). In contrast, by neither Coomassie nor silver staining could we detect differences between outer membrane preparations of wild-type and *ΔompA* strains of *Y. pestis* (*data not shown*). Therefore, OmpA in *Y. pestis* appears to be expressed at lower levels than in *E. coli.* However, despite these differences in expression levels, we found by Northern and Western analysis, that in *Y. pestis*, as has been shown in *E. coli*, the expression levels of *ompA* transcript and OmpA protein are higher in growing cells compared to stationary phase cells (*data not shown*).

A contributing factor to the growth phase regulation of OmpA expression in *E. coli* involves the negative regulator small RNA (sRNA) MicA, which is expressed at higher levels in stationary phase cells (Smith et al., 2007). The *Y. pestis* KIM5 genome harbors a sequence that resembles the sequence of the *E. coli* MicA. To determine whether *Y. pestis* expresses this MicA-like gene, we analyzed RNA from the wild-type strain as well as a strain in which this putative gene had been deleted (*ΔmicA*). We detected a transcript of the expected length in wild-type, but not from the *ΔmicA* strain (Fig. 6A). In *E. coli* the level of MicA transcripts are higher in strains lacking the ribonuclease polynucleotide phosphorylase (PNP) (Andrade & Arraiano, 2008). Similarly, in the *Y. pestis* Δ*pnp* strain (Rosenzweig *et al*., 2005), we also observed higher levels of the MicA transcript (Fig. 6A). We also found that in *Y. pestis*, again, as has been described for *E. coli*, the levels of MicA are higher in stationary phase cells compared to log-phase cells (*data not shown*). These data indicate that *Y. pestis* expresses a functional MicA that likely contributes to the growth-phase regulation of OmpA expression.

To test whether MicA overexpression would affect *Y. pestis* infection of macrophages, we cloned the MicA-encoding sequence in a high-copy plasmid. The strain carrying the MicA-

encoding plasmid displayed increased levels of MicA transcripts and, as expected, reduced levels of OmpA protein (Fig. 6B). The MicA overexpressing strain also had a much reduced infectivity compared to the strain harboring the vector control plasmid (Fig. 6C). This effect was specific for MicA since overexpression of other sRNAs did not impact infectivity (*data not shown*). This experiment does not necessarily mean that MicA regulates OmpA levels during infection only that, like in *E. coli*, there is an inverse relationship between MicA and OmpA levels. Nonetheless, the phenotypic similarly between the MicA overexpression strain and the *ΔompA* mutant strain further indicates the importance of OmpA for *Y. pestis* infection.

2.4. Animal infections corroborate in vitro findings

In vivo competition assays provide a sensitive measure of the extent of virulence attenuation caused by a given mutation. A mouse BSL2 model of pneumonic plague (Galván *et al.* 2010) was used to compare the virulence of wild-type and Δ*ompA Y. pestis* strains. C57BL/6 mice were inoculated intranasally with equal numbers of *Y. pestis* KIM5 (Pgm-) and an isogenic *ompA* deletion strain. Bacteria were recovered from the lungs, spleen and liver at 18 h, 36 h and 60 h post-infection. Analysis of the ratio of wild type CFUs to Δ*ompA* CFUs recovered from the lungs, spleen and liver (Fig. 7) revealed that the ompA mutant was significantly out competed by the wild-type parent strain. These findings indicate that the *ompA* mutant is attenuated for virulence in the mouse BSL2 pneumonic plague model and is consistent with the *in vitro*-derived findings showing that OmpA is important for the intracellular survival of *Y. pestis*.

This is the first report of OmpA having a role in the pathogenesis of the plague bacterium *Y. pestis*. Our results fit with the developing theme that OmpA can potentially serve a variety of functions for Gram-negative pathogens. For *Y. pestis* (and *Y. pseudotuberculosis*) OmpA enhances intracellular survival (possibly by conferring resistance to antimicrobial peptides), but in contrast to other pathogens, serves no detectable role in adherence, invasion, or serum resistance.

3. Methods

3.1. Bacterial strains and in vitro infection assays

The parental *Y. pestis* KIM5-3001 and *ΔyopB*, *Δpnp*, and *Δail* strains have been described (Linder *et al.*, 1990; Rosenzweig *et al.*, 2005; Bartra *et al.*, 2008). KIM5-3001 derivative strains were made with deletions in *ompA* (y2735; condons 14–340), *ompC* (y2966; codons 4–370), *ompF* (y2759; codons 1–326), and *ompN* (y2983; codons 8–335) using *in vivo* lambda red recombination (Datsenko and Wanner 2000). The parental *Y. pseudotuberculosis* YPIII/pIB102 strain (Bolin *et al.* 1982) was similarly deleted in *ompA*. For the TraSH-based analysis, about 1.5×10^7 murine macrophage-like RAW 264.7 were infected with the pooled *Y. pestis* transposon mutants (described below) at a MOI of 2 for 14 hrs. The infection assay with engineered strains of *Y. pestis* (Fig. 3) was performed as described (Rosenszweig et al., 2005). The invasion assay using *Y. pestis* (Fig. 4) involved infecting RAW 264.7 cells at a MOI of 10. After a 45 min attachment period, gentamicin was added at a concentration of 8 μg/ml which, after an additional hour, was replaced with media containing 4 μg/ml gentamicin. At the indicated times the infected cells were lysed with water which was plated for colony counts. For, the invasion assay using *Y. pseudotuberculosis* (Fig. 5) RAW 264.7 cells were infected with a MOI of 10 and 45 min later the media was removed and replaced by media containing 0.5 μg/ml gentamicin. At the indicated times infected cells were lysed with water and colony forming units determined by plating.

3.2. TraSH-based screening

A mutant library was generated by introducing a miniTn5 construct into *Y. pestis* KIM5 by electroporation. The miniTn5 was engineered to contain one outward facing T7 promoter at each end by joining a T7p-containing oligonucleotide to the EZ-Tn5™ <T7/KAN-2> DNA (Epicentre, Madison, WI) by PCR. The library of mutants was used in the infection experiment described above. The mutant bacteria that survived infection were collected to make genomic DNA. Labeled RNA probes were generated from gDNA samples and were used to hybridize a genome tiling microarray containing 40-mer oligonucleotides (Affimetrix, Santa Clara, CA), at the DNA microarray facility of Ocean Bridge Bioscience Inc., Palm Beach Gardens, FL. Standard hybridization, array scanning and data normalization procedures were used. In order to analyze gene-specific hybridization signals, we first identified those genes for which there is at least one probe that hybridizes only within the gene's open reading frame. We have constructed a list of genes that are unimportant for infection by excluding the known important genes, and used this list as negative control for the identification of important genes. The raw data were pre-processed through median polishing of the LOESS normalized log2 fold changes. The pre-processed data were analyzed for each qualified gene using Welch's t-test. Genes that are important for infection were identified if the gene's specific signals are significantly lowered after infection as compared to unimportant genes control ($p < 0.05$).

3.3. Mice infection

The virulence defect of the Δ*ompA* strain was studied in a co-infection experiment with the parental strain KIM5 in a mouse BSL-2 model of pneumonic plague (Galvan *et al*., 2010). Eight weeks old female C57BL/6 mice were purchased from the Jackson Laboratories. The mice were provided food and fresh water *ad libitum* during the experiments, which were performed according to the guidelines of the University of Pennsylvania Institutional Animal Care and Use Committee. Bacteria were grown in BHI at 26°C overnight, diluted 1:10 in fresh BHI containing 2.5 mM $CaCl_2$, and cultured overnight at 37°C. The bacterial cells were pelleted by centrifugation, washed and re-suspended in PBS to A_{600} of 0.2 (approximately 2×10^7 CFU/ml). Equal volumes of the parental and Δ*ompA* strain suspensions were mixed and 10-fold serially diluted in PBS to prepare the inoculum. Bacterial counts were determined on TB agar plates (Galvan et al., 2008). Mice were given three injection of iron dextran (Sigma; 4 mg each) intra-peritoneally at 24 h intervals. 3 h after the first injection with iron dextran, mice were anesthetized (ketamine/xylazin 100/10mg/kg body weight, i.p.) and infected by intra-nasal instillation with 25 μl of the bacterial inoculum containing 4×10^3 CFU of each strain. Lungs, livers and spleens were surgically removed at 18, 36 and 60 h post infection and homogenized in 5 ml sterile PBS by using a Stomacher Lab Blender (Seward Medical Limited). CFU/organ were determined by plating serial dilutions onto TB agar plates containing streptomycin or kanamycin. CFUs for the parental strain KIM5 were calculated by subtracting the Δ*ompA* CFUs (kanamycin plates) from the total CFUs (streptomycin plates). Competitive index (C.I.) scores were calculated as the ratio of the KIM5 CFUs to the Δ*ompA* CFUs. Statistical significance was determined by the paired *t*-test.

Acknowledgments

The gene screening work was supported by University of South Florida Center for Biological Defense, funded by U.S. Army Research, Development and Engineering Command (RDECOM), Contract W911SR-07-C-0084 (ZL). This work was also supported in part by Public Health Service grants AI076695 (DMS), AI50552 (GVP), and AI53459 (KS) from the National Institute of Allergy and Infectious Diseases.

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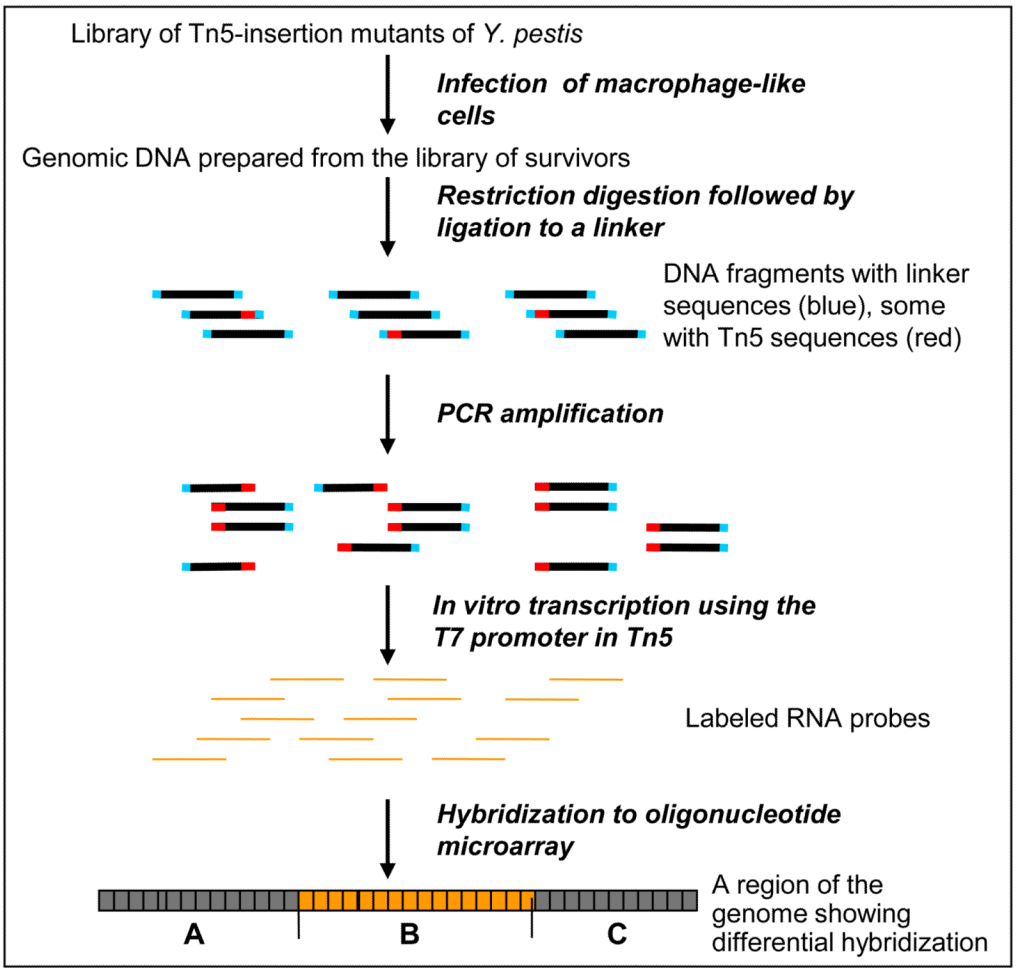

Fig. 1. Overview of TraSH-based approach for the identification of genes essential for infectivity of *Yersinia pestis*

A library of *Y. pestis* mutants was used to infect murine RAW 264.7 macrophage-like cells. The mutants that survived infection were collected and genomic DNA was prepared. The DNA was digested with a restriction enzyme that cleaves once in the middle of Tn5, and the fragments were ligated to a linker DNA. Tn5-containing sequences were amplified by PCR using Tn5 and linker specific primers. Labeled RNA probes were made from the T7 promoters at the termini of Tn5, and were used to hybridize a custom genome tiling oligonucleotide microarray. Oligonucleotides are differentially hybridized depending on the abundance of the RNA probes corresponding to that region of the genome. Genes that are potentially important for infection can are indicated by their lower hybridization signals from the library survived infection compared to those from the library grown in tissue culture medium alone.

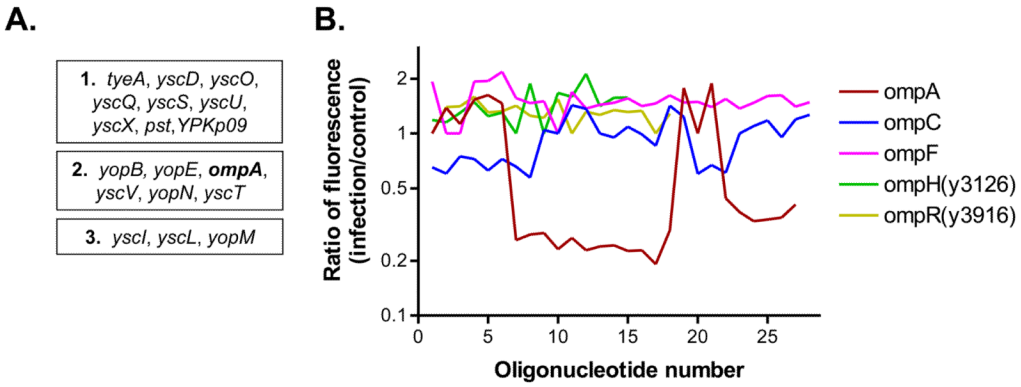

Fig. 2. The *Y. pestis ompA* locus (y2735) encodes a pro-survival factor

(A) The 17 infection-promoting loci encoded on the extracellular plasmids pCD1 and pPCP1, together with the *ompA* locus, that were identified in the TraSH screen were arranged into three groups according to their respective infection/control ratios: (1) those ORFs that had a ratio lower than 0.5; (2) those ORFs with a ratio of between 0.5 and 0.7; and (3) those ORFs with a ratio higher than 0.7 but significantly lower than that of non-essential genes. **(B)** Fluorescence associated with several *omp* or *omp* regulatory gene regions of the microarray. There is at least one probe specific for each of the indicated genes. The relative reduction of signal in the *ompA* sequences from the infection population indicates the relative underrepresentation of *ompA*(Tn+) mutants following infection compared to population propagated in the absence of macrophages.

FIG. 3A

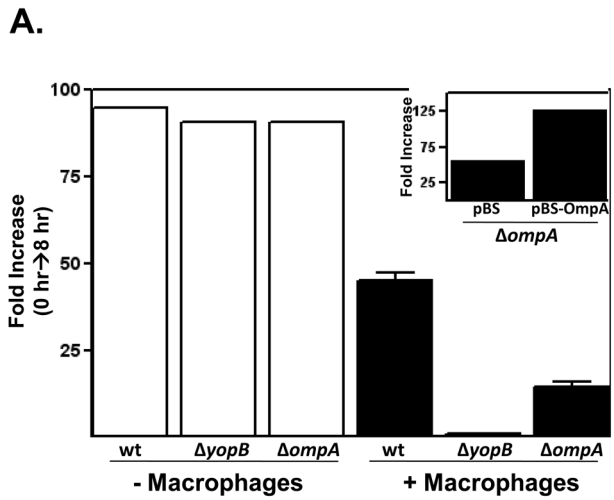

FIG. 3B, C

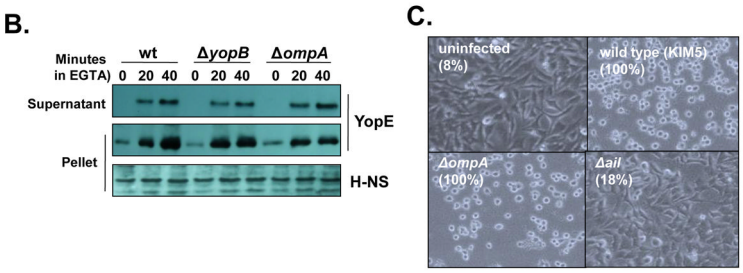

Fig. 3. The *Y. pestis* OmpA promotes infectivity independently of the T3SS

(A) Empty wells (-macrophage) or wells containing RAW 264.7 cells were infected with *Y. pestis* KIM5, Δ*yopB* and Δ*ompA* strains at a MOI of 1. Following a 30 minute attachment period, unattached bacteria were removed, and the number of cell-associated bacteria was determined by plating at the 0 hour and 8 hour timepoints. Three independent wells per strain were analyzed and the average fold-increase in the number of bacteria recovered for each strain over the 8 hour infection period is shown. (*inset*) The infectivity of the *Y. pestis* Δ*ompA* strain was restored by a plasmid-encoded OmpA. **(B)** Yop secretion by the *Y. pestis* KIM5, Δ*yopB* and Δ*ompA* strains. Strains were grown to mid-log phase and then EGTA was added to induce Yop secretion. YopE levels in both whole cells and supernatant fractions were determined by immunoblotting. H-NS levels were analyzed as a loading control. **(C)** HeLa cells were left uninfected or infected with wild-type *Y. pestis* KIM5, Δ*ompA* or Δ*ail* strains at an MOI of 30 and photographed 2 hours after infection. Cell rounding (cytotoxicity) was quantified by counting all cells (N 200) in three different fields and the percentage is shown in parenthesis.

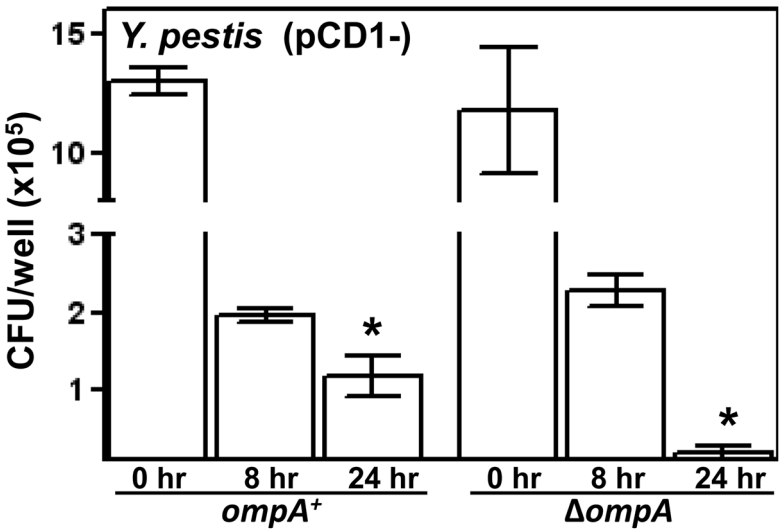

Fig. 4. OmpA promotes intracellular survival of *Y. pestis*

Wild-type and Δ*ompA* strains of *Y. pestis* lacking the T3SS encoding virulence plasmid pCD1 were added to RAW 264.7 cells at a MOI of 10. Following a 45-min attachment/invasion period gentamicin was added to each well. After 0, 8 and 24 hrs of infection the number of cell-associated bacteria per well was determined by viable plating. One of three independent experiments is shown. (* $P < 0.05$)

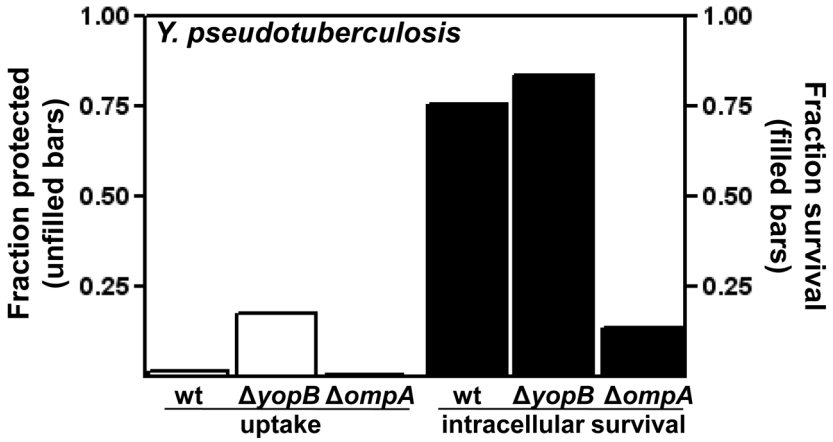

Fig. 5. OmpA promotes intracellular survival of *Y. pseudotuberculosis*

Wild-type *Y. pseudotuberculosis* YPIII, Δ*yopB* and Δ*ompA strains* were added to RAW 264.7 cells at a MOI of 10. After a 45-min attachment period excess bacteria were removed and gentamicin was added to half of the wells. 'Uptake' (open bars) was determined by dividing the number of CFUs recovered from the gentamicin-containing wells by the number of CFUs recovered from the untreated wells following an additional 1.5 hrs of infection. 'Intracellular survival' (closed bars) was determined by dividing the number of CFUs recovered from gentamicin-containing wells after 5 hrs of infection by the number of CFUs recovered from the gentamicin-containing wells after 1.5 hours of infection. One of three independent experiments is shown.

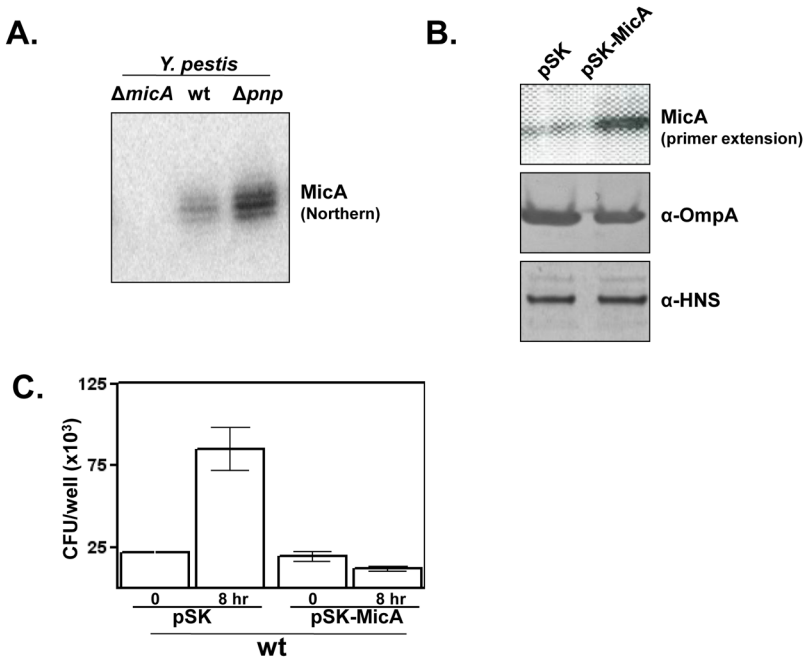

Fig. 6. MicA expression in *Y. pestis* and functional consequences of its overexpression

(A) Total RNA was isolated from the indicated *Y. pestis* strains and analyzed by Northen blot analysis using a MicA specific probe. **(B)** Either the empty vector or MicA-encoding plasmids were transformed into wild-type *Y. pestis* KIM5 and the levels of MicA transcript and OmpA protein levels in the resulting transformant strains were measured by primer extension and western analysis, respectively (α-HNS: loading control). **(C)** RAW 264.7 cells were infected with *Y. pestis* transformed with either empty vector or the MicA-encoding plasmids and analyzed as described in Fig. 1. One of three independent experiments is shown.

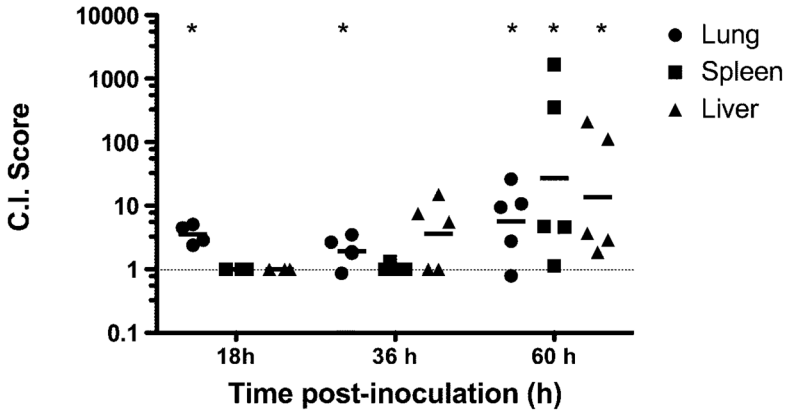

Fig. 7. Wild type *Y. pestis* out competes an isogenic Δ*ompA* mutant in a mouse BSL2 model of pneumonic plague

Y. pestis KIM5 and the isogenic Δ*ompA* mutant were mixed 1:1 and inoculated intranasally into groups of five C57BL/6 mice. Bacteria were recovered from the lungs, liver and spleen at 18 h, 36 h and 60 h post-infection. The competitive index (CI) was calculated as the ratio of the CFU of wild type *Y. pestis* to the CFU of the Δ*ompA* mutant recovered from the lungs, liver and spleen of each mouse. Statistically significant reductions in organ colonization by the mutant strain are labeled by asterisks (paired t-test, $P < 0.05$).