Varying dependency of periplasmic peptidyl-prolyl cis/trans isomerases in promoting Yersinia pseudotuberculosis stress tolerance and pathogenicity

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Varying dependency of periplasmic peptidyl-prolyl *cis/trans* isomerases in promoting *Yersinia pseudotuberculosis* stress tolerance and pathogenicity

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Short title: Periplasmic PPIases from *Yersinia*

Abbreviations used: PPIase(s), periplasmic peptidyl-prolyl *cis/trans* isomerase(s); ROIs, reactive oxygen intermediates; ID_{50}, 50% infectious dose; LPS, lipopolysaccharide; CsA, cyclosporin A; FKBP, FK506 binding proteins; YSA, *Yersinia* selective agar; SDS, sodium dodecyl sulfate; CFU, colony forming units; IPTG, Isopropyl-β-D-thio-galactoside; MALDI-TOF, Matrix assisted laser desorption ionization time of flight mass spectrometry; FA, fatty acids; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin.

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SYNOPSIS

Periplasmic peptidyl-prolyl *cis/trans* isomerases (PPIases) catalyze the *cis/trans* isomerization of peptidyl-prolyl bonds, which is a rate-limiting step during protein folding. We demonstrate that the *surA, ppiA, ppiD fkpA* and *fklB* alleles each encode a periplasmic PPIase in the bacterial pathogen *Yersinia pseudotuberculosis*. Of these, four were purified to homogeneity. Purified SurA, FkpA and FklB, but not PpiD, displayed detectable PPIase activity *in vitro*. Significantly, only *Y. pseudotuberculosis* lacking *surA* caused drastic alterations to the outer membrane protein profile and fatty acid composition. They also exhibited aberrant cellular morphology, leaking LPS into the extracellular environment. The SurA PPIase is therefore most critical for maintaining *Y. pseudotuberculosis* envelope integrity during routine culturing. On the other hand, bacteria lacking either *surA* or all of the genes *ppiA, ppiD, fkpA* and *fklB*, were sensitive to hydrogen peroxide and were attenuated in mice infections. Thus, *Y. pseudotuberculosis* exhibits both SurA-dependent and SurA-independent requirements for periplasmic PPIase activity to ensure *in vivo* survival and a full virulence effect in a mammalian host.

Key words: membrane biogenesis, chaperone, protein folding, immunosuppressant, survival, infection.

INTRODUCTION

PPIases catalyze the *cis/trans* isomerization of peptidyl-prolyl bonds in peptide and protein substrates [1, 2]. Isomerization of proline residues is a rate-limiting step in protein folding, underlying the ubiquitous presence of PPIases in all kingdoms of life and in different cellular compartments [2]. Three major classes of PPIases exist: the Cyclophilins, which bind the cyclosporin A (CsA) immunosuppressive drug; the FK506 binding proteins (FKBP), which bind the FK506 immunosuppressant; and the PPIases that have sequence similarity with Parvulin from the *E. coli* cytoplasm [1, 2].

The bacterial periplasm contains all three PPIase classes. In *E. coli*, four definitive periplasmic PPIases have been described; SurA, PpiA, PpiD and FkpA [3, 4]. Another newly identified PPIase is denoted FklB, and might constitute the newest member of the periplasmic PPIase family [5]. SurA and PpiD belong to the Parvulin class of PPIases; PpiA is a Cyclophilin, while FkpA and FklB are related to the FKBP family. SurA is well studied; most bacteria lacking *surA* are more susceptible to detergents, antibiotics and hydrophobic dyes suggestive of a compromised outer membrane barrier function (reviewed in [6]). On the other hand, *in vitro* phenotypic defects in bacteria lacking the other periplasmic PPIases are seldom reported.

Yersinia pseudotuberculosis is an environmental bacterium associated with sporadic enteric disease outbreaks known as yersiniosis caused by the ingestion of contaminated food or fluids [7]. While this disease causes gastrointestinal discomfort, it is usually self-limiting and rarely associated with systemic disease. It is also a close relative of another enteric pathogen *Y. enterocolitica* [7] as well as the infamous plague-causing pathogen *Y. pestis* [8].

In this study, *in silico* analysis revealed all these five putative periplasmic PPIases in the *Y. pseudotuberculosis* YPIII genome sequence (US DOE Joint Genome Institute; GenBank accession number is CP000950) based on their amino acid sequence identity to their homologues in *E. coli* K12 [9]. We purified SurA, PpiD, FkpA and FklB and performed a subsequent biochemical analysis of their PPIase activity. Furthermore, we created cumulative *in cis* deletions of the 5 periplasmic PPIase genes in *Y. pseudotuberculosis* and examined their contribution to envelope integrity and survival during exposure to extracytoplasmic stress, reactive oxygen intermediates (ROIs), low pH and *in vivo* mouse infections.

EXPERIMENTAL

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are shown in Table 1.

Growth conditions

Unless stated otherwise, bacteria were cultivated routinely in Luria-Bertani (LB) agar or broth at either 26 °C (*Y. pseudotuberculosis*) or 37 °C (*E. coli*) with aeration. When required, antibiotics were used at the following final concentration: 50 µg/mL kanamycin, 25 µg/mL chloramphenicol and 100 µg/mL carbenicillin.

To assay for the viability of PPIase mutants in the presence of bile salts or detergents, overnight cultures of *Y. pseudotuberculosis* grown in LB broth at 26 °C were sub-cultured (0.1 volume) into 3mL fresh media. The bacteria were allowed to grow for a further 2 h before being serially diluted and then plated on LB agar, *Yersinia* selective agar rich in bile salts (BD

Stockholm, Sweden) or LB agar supplemented with 0.0125 % (v/v) SDS or 0.25 % (v/v) sodium deoxycholate. Colony forming units (CFU) were counted after 48 h of growth.

An antibiotic sensitivity assay involved mixing 100 µL from overnight cultures of *Y. pseudotuberculosis* with 10 mL molten 0.6 % soft-agar that was then poured over LB agar plates. Vancomycin and bacitracin-impregnated discs (Oxoid, Hampshire, England) were then overlaid on the agar surface. After 24 h incubation at 26 °C, the diameter of the inhibitory zone around the discs was measured.

To assay for stationary phase survival, *Y. pseudotuberculosis* overnight cultures were sub-cultured (0.1 volume) into fresh LB broth and grown at 26 °C to mid-log phase. These bacteria were again back-diluted (0.001 volume) into 50 mL LB broth. During 6 days incubation, samples were taken at daily intervals, serially diluted and then plated on LB agar. CFU were determined after 48 h.

To test sensitivity to menadione and hydrogen peroxide, *Y. pseudotuberculosis* overnight cultures were sub-cultured (0.005 volume) into fresh LB broth and grown at 26 °C for 1 h. Samples were serially diluted and then plated on LB agar supplemented with 200 µM menadione or 1 mM hydrogen peroxide. CFU were counted after 48 h of growth.

For the pH testing, overnight cultures of *Yersinia* were subcultured in LB broth (0.1 volume) buffered with 100 mM citrate pH 3 or pH 5. Bacteria were grown at 26 for 6 hours. Samples were taken at 0, 3 and 6 hours. After serial dilution, samples were plated on LB-agar. CFU were counted after 48 hours. LB broth buffered with 20 mM potassium phosphate pH 7 was used as a control.

Generation of mutants

Overlap PCR with template DNA from *Y. pseudotuberculosis* YPIII/pIB102 was used to generate mutated alleles for constructing the full-length in-frame deletion mutants. The primer pairs used to generate each mutation are listed in Supplementary Table S1. Amplified fragments were cloned into the suicide mutagenesis vector, pDM4. *E. coli* S17-1λ*pir* harbouring each specific mutagenesis vector was used in conjugal mating experiments with *Y. pseudotuberculosis*. Selection for the appropriate allelic exchange events giving rise to the desired *Y. pseudotuberculosis* mutants used our conventional sucrose sensitivity methodology [10].

Over-expression and purification of recombinant PPIases

PPIase genes were PCR amplified from the *Y. pseudotuberculosis* YPIII/pIB102 genome using the primer pairs listed in Supplementary Table S1. Codons 2 to 105 of *ppiD* were omitted to avoid sequences encoding for a putative trans-membrane region. The DNA fragments were cloned in pET30a(+) (Novagen) generating PPIase gene sequences in frame with the 5´ His-tag resident in the plasmid. Recombinant PPIases were over-expressed in *E. coli* BL21 grown to logarithmic phase (OD_{600} of 0.5) in 250 mL of LB broth. Upon addition of 0.4 mM IPTG bacteria were cultured for a further 3 h. Bacteria were washed with 50 mL lysis buffer (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 30 mM Imidazole) and concentrated 10-fold in this buffer containing lysozyme (1 mg/ml). Afterwards, bacteria were lysed by sonication and soluble lysates clarified by centrifugation (32,000 g, 1 h) and passaged through a 0.2 µm filter prior to protein purification by affinity chromatography on Ni^{2+} agarose columns (GE Healthcare, Sweden) using the AKTA purifier (GE Healthcare). Columns were washed with 50 mL lysis buffer and the proteins eluted with 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 1M Imidazole using

a linear gradient. Pooled eluted fractions were dialyzed overnight against 20 mM Tris-HCl pH 8, 50 mM NaCl. Protein purity was assessed by SDS-PAGE and Coomassie Blue staining. Protein concentration was determined using BCA protein assay kit (Pierce, Rockford, USA).

PPIase assay

The PPIase activity was performed in 50 mM Tris-HCl, pH 8 at 10 °C using the protease-coupled assay [11]. The peptide substrate, Suc-Ala-Leu-Pro-Phe-pNa, was purchased from Bachem (Switzerland). Nitroaniline appearance due to α-chymotrypsin cleavage of the peptide *trans*-isomer was followed at 390 nm for 3 min with a Beckman Coulter DU 730 Life Science UV/Vis spectrophotometer. First-order rate constants (k_{obs}) were derived by fitting reaction progress-curves to a first-order rate equation ($y_{390} = y_0 + a(1-e^{-kt})$, where k is the rate constant, y_0 is the absorbance at time 0). k_{cat}/K_m was calculated from the plot of k_{obs} versus PPIase concentration.

The PPIase inhibition assay used stock solutions of each immunosuppressant CsA, FK506 or rampamycin (LC laboratories, Massachusetts) prepared in ethanol/water (1:1, v/v). PPIases (0.1 μM) were incubated with various concentrations of drug on ice for 7 min prior to PPIase activity measurements. The respective rate constants in the presence of the inhibitors were derived by fitting reaction progress-curves to the first-order rate equation.

Whole bacterial lysates and cellular fractionation

To prepare whole bacterial cell lysates, overnight cultures of *Y. pseudotuberculosis* were grown at 26 °C and 37 °C. After standardization of the optical density at 600 nm and centrifugation (5,000 g, 10 min), cells were lysed in SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2 % SDS, 10 % glycerol, 5 % β-mercaptoethanol and 0.1 % bromophenol blue) and heat inactivated.

For fractionation experiments, bacteria grown to logarithmic phase in 200 mL LB broth at 26 °C were resuspended in 30 mM Tris-HCl, pH 8, 20 % sucrose at 80 mL per gram wet weight. After addition of 500 mM EDTA drop wise to an end concentration of 1 mM and a 10 min incubation on ice with gentle agitation, cells were harvested (8,000 g, 20 min, 4 °C) and the pellet resuspended in 500 μL of ice-cold 5 mM $MgSO_4$. Following agitation for 1 h at 4 °C, the periplasmic fraction (supernatant) was collected (8,000 g, 20 min) and subsequently mixed with SDS sample buffer. The pellet was resuspended in 500 μL 100 mM Tris-HCl, pH 8, 10 mM EDTA and the osmotic-shocked cells were lysed by three freeze-thaw cycles and a 1 min sonication. The cytoplasmic fraction (supernatant) was recovered (22,000 g, 30 min) and mixed with SDS sample buffer. The remaining pellet (crude membrane fraction) was washed with 500 μL 20 mM K_2PO_4 (pH 7). To separate inner membrane from outer membrane, the pellet was resuspended in 100 μL of 0.5% sarcosyl in 20 mM K_2PO_4 (pH 7). After incubation at room temperature for 30 min, the inner membrane (soluble fraction) was collected (22,000 g) and treated with SDS sample buffer. The outer membrane (pellet) was washed once with 100 μL 0.5% sarcosyl in 20 mM K_2PO_4 (pH 7) and resuspended in SDS sample buffer. Following separation by 12 % SDS-PAGE, proteins were transferred to PVDF membrane (Millipore, Bedford, USA) and identified with rabbit polyclonal antibodies raised against individually purified recombinant PPIase proteins (Agrisera AB, Vännäs, Sweden) as approved by the Animal Ethics Committee of Umeå University (ethics number A102/09).

Mass spectrometry and protein identification

Sample preparation, mass spectrometry and protein identification was based on previously described procedures [12]. A protein ID was considered valid if it was top-most on the putative hit list generated by the MASCOT search engine (http://www.matrixscience.com) and with a probability based Mowse score above the significance threshold ($p<0.05$; *vide infra*).

Isolation of LPS

To isolate LPS liberated free from *Y. pseudotuberculosis* grown overnight in 200 mL LB broth at 26 °C, we adapted a method for outer membrane vesicle purification [13] by including a terminal proteinase K digestion step. Briefly, to pellets resuspended in 20 mM Tris-HCl, pH 8, SDS was added to a final concentration of 0.1 % and samples were heated for 3 min at 95 °C to denature proteins. Final concentrations of 5 mM $CaCl_2$ and 7 µg proteinase K were added and the samples incubated overnight at 37 °C. Following treatment with sample buffer, isolated LPS were analyzed by 13.5 % SDS-PAGE and silver staining (Fermentas Life Sciences).

Crude total cellular LPS was prepared from stationary phase *Y. pseudotuberculosis* grown in LB broth at 26 °C. Bacterial pellets were collected by brief centrifugation, resuspended in sample buffer and lysed at 95 °C for 10 min. Final concentrations of 5 mM $CaCl_2$ and 7 µg proteinase K were added and the samples incubated overnight at 37 °C. LPS analysis was performed by 13.5 % SDS-PAGE and silver staining.

Isolation and characterization of cellular fatty acids and phospholipids

The analysis of fatty acid and phospholipid composition of *Y. pseudotuberculosis* YPIII was performed commercially within the quality assured laboratories at Mylnefield Lipid Analysis (Dundee, UK) essentially using the method of Folch et al [14].

Transmission electron microscopy (TEM)

Y. pseudotuberculosis was harvested from LA plates after 48 h. Bacteria were resuspended in 10 mM Tris-HCl buffer, pH 7.4, 10 mM MgCl and allowed to adhere to formvar-coated grids for 3 min at room temperature. Following negative staining with 1% sodiumsilicotungstate, images of bacteria were acquired using a Jeol JEM 1230 electron microscope at 40,000x magnification.

Mouse infections

Infections were performed in accordance with the guidelines of the Animal Ethics Committee of Umeå University (ethics number A81-08). Groups of five female BALB/c mice (Scanbur, Denmark) of similar age (8 to 9 weeks) and weight (~18 g), were injected intraperitoneally with 0.1 ml of different dilutions of *Y. pseudotuberculosis* parent and mutant bacteria that had been grown overnight in LB broth at 26°C. Pelleted bacteria were serially diluted to 10^7, 10^6 and 10^5 CFU/ml in PBS. Infection was monitored for 14 days post-injection. The 50% infectious dose (ID_{50}) was determined by the Reed-Muench method [15], with positivity of infection reflected by symptoms of ruffled fur, weight loss, listlessness and diarrhoea. Infected mice showing symptoms of a terminal infection were immediately euthanized.

RESULTS

Identification of *Y. pseudotuberculosis* periplasmic PPIases

Amino acid sequence alignments with their homologues in *E. coli* K12 revealed five potential periplasmic PPIases in the *Y. pseudotuberculosis* YPIII genome: *surA* (NCBI annotation

YPK_3571), *ppiD* (YPK_3230), *ppiA* (YPK_0242), *fkpA* (YPK_0270), and *fklB* (YPK_3778) (Figure 1A). Their amino acid sequences were at least 99 % identical to their homologues in *Y. pestis,* between 85 to 98 % in *Y. enterocolitica* and 58 to 79 % in *E. coli* (Figure 1B). Based upon BLAST motif searches (Figure 1C), SurA (434 amino acids) is comprised of four domains: a unique N-terminal domain, two Parvulin domains and a C-terminal domain possessing an unknown fold. PpiD (628 amino acids) contains three domains: a trans-membrane domain, an N-terminal domain that is similar to that of SurA, and a Parvulin domain. In addition, the protein contains a long C-terminal region of unknown fold. PpiA (189 amino acids) possesses a Cyclophilin domain, while FkpA (266 amino acids) and FklB (206 amino acids) consist of two domains: N- and a C-terminal domain both displaying (FKBP) consensus.

Production and purification of *Y. pseudotuberculosis* periplasmic PPIases

surA, ppiA, ppiD, fkpA and fklB genes were PCR amplified from *Y. pseudotuberculosis* YPIII/pIB102 genome and cloned into pET-30a(+). IPTG induction of protein expression in *E. coli* BL21 cells resulted in soluble forms of recombinant SurA, PpiD, FkpA and FklB, whereas PpiA was produced as an insoluble protein (data not shown). Varying IPTG concentration or induction temperature did not improve the solubility of recombinant PpiA (data not shown). Soluble forms of SurA, PpiD, FkpA and FklB were purified from *E. coli* cell-lysates using nickel affinity chromatography and protein purity was analyzed by SDS-PAGE (Figure 2). This one-step purification process provided very pure protein that migrated to about 52 kDa, 74 kDa, 33 kDa and 27 kDa on SDS-PAGE, corresponding to the apparent molecular masses of recombinant SurA, PpiD, FkpA and FklB, respectively. Protein yield of about 3 mg was obtained from a 250 mL culture for each protein.

SurA, FkpA and FklB exhibit detectable PPIase activity

Having purified recombinant SurA, PpiD, FkpA and FklB, we performed a rarely executed comparative assessment of their individual PPIase activity using a α-chymotrypsin-coupled spectrophotometric assay that monitors the *cis/trans* isomerization of the peptidyl-prolyl bond in the tetrapeptide Suc-Ala-Leu-Pro-Phe-pNa. This assay is based on the fact that α-chymotrypsin can hydrolyze the 4-nitroanilide amide bond in the tetrapeptide only when the Leu-Pro bond is in the *trans*-conformation. SurA, FkpA and FklB displayed PPIase activity (Figure 3A). No detectable activity was observed with PpiD, consistent with an earlier report concerning *E. coli* PpiD [16] or for the two-component response regulator, CpxR, which was used as a negative control (Figure 3A). SurA PPIase activity was low in comparison to the robust activity of FkpA and FklB. The lower intrinsic SurA PPIase activity has also been observed for *E. coli* SurA [17, 18]. Furthermore, the PPIase activity of SurA, FkpA and FklB was concentration dependent (Figure 3B). Under our assay conditions, the catalytic efficiencies (k_{cat}/K_m) of FklB (1.1×10^6 $M^{-1}s^{-1}$) was highest, followed by that of FkpA (2.6×10^5 $M^{-1}s^{-1}$), whereas SurA (4.7×10^4 $M^{-1}s^{-1}$) has the least specificity for the tetrapeptide substrate. FklB and FkpA catalytic efficiencies are comparable to other biochemically verified FKBPs [19, 20].

To confirm the presence of an intrinsic PPIase activity in the *Y. pseudotuberculosis* periplasm, we isolated periplasmic extracts from the parental bacteria and a quintuple mutant (Δ*ppiA* Δ*ppiD* Δ*fkpA* Δ*fklB* Δ*surA*) lacking the five periplasmic PPIase genes. These extracts were assayed for PPIase activity using the tetrapeptide Suc-Ala-Leu-Pro-Phe-pNa. Periplasmic extract from the parental bacteria was active in *cis/trans* isomerization of peptidy-prolyl bond of the tetrapeptide, whereas no PPIase activity was detected in the periplasmic extract of the quintuple mutant

(Supplementary Figure S1). We therefore considered that a lack of PPIase activity in the periplasmic fraction of the Δ*ppiA* Δ*ppiD* Δ*fkpA* Δ*fklB* Δ*surA* quintuple mutant was indicative of an important *in vivo* role for these periplasmic PPIases in maintaining optimal *Y. pseudotuberculosis* fitness.

PPIase inhibition by immunosuppressive drugs

PPIases were first discovered based on their high affinity to bind immunosuppressive drugs. To investigate the effects of CsA, FK506 and rampamycin immunosuppressants on SurA, FkpA and FklB, 0.1 μM of protein was incubated with various concentrations of the drugs, and then assayed for remaining PPIase activity. CsA, FK506 and rampamycin showed no detectable effect on SurA PPIase activity, even at excessive concentrations (10 μM for CsA and 0.5 μM for FK506 and rampamycin) (Figure 4A and data not shown). This is consistent with SurA being classified as a Parvulin-like PPIase. However, FkpA (Figure 4B) and FklB (Figure 4C) were strongly inhibited by FK506 and rampamycin in a dose-dependent manner. Rampamycin was more effective at causing complete inhibition of FkpA and FklB activity with an IC_{50} of 19 nM and 51 nM, respectively; this compared to an IC_{50} of 43 nM and 119 nM needed for FK506 to inhibit FkpA and FklB activity, respectively. These data reflect the higher affinity of rampamycin to FKBPs [21]. As anticipated, CsA failed to diminish the PPIase activity of FkpA or FklB (Figure 4B and 4C).

Sub-cellular localization of SurA, PpiD, FkpA and FklB in *Y. pseudotuberculosis*

Protein prediction algorithms indicate the presence of signal peptides in SurA and FkpA as well as a trans-membrane domain in PpiD (data not shown). This implies that these proteins are likely to be localized in the bacterial envelope. However, no related information is available for FklB. We therefore utilized each recombinant purified PPIase as antigen to generate specific polyclonal rabbit antibodies. These were then used to experimentally analyze the sub-cellular localization of SurA, PpiD, FkpA and FklB in the same *Y. pseudotuberculosis* whole cell-lysate that was separated into outer membrane, periplasmic, inner membrane and cytoplasmic fractions. To control fractionation purity, TolC, MBP, FtsH and H-NS were used as outer membrane, periplasmic, inner membrane and cytoplasmic markers, respectively. All of the PPIases could be detected in the whole cell-lysate (Figure 5). SurA and FkpA were enriched in the periplasmic fraction, while PpiD was enriched in the inner membrane fraction. These data are consistent with their repesctive localizations in *E. coli* [22, 23]. However, contrary to a previous study that identified FklB in the periplasm using indirect detection with the Mip antibody [5], our anti-FklB antibodies identified FklB exclusively in the inner membrane.

Peptidoglycan-associated lipoprotein (Pal) and OmpA are among several outer membrane proteins reduced in *surA* mutants

In *E. coli*, SurA functions as a periplasmic folding factor of various outer membrane proteins (reviewed in [6]). To study effects of loss of *surA* on outer membrane proteins in *Y. pseudotuberculosis*, outer membrane protein profiles of parental, *surA* single (Δ*surA*), quadruple (Δ*ppiA* Δ*ppiD* Δ*fkpA* Δ*fklB*) and quintuple (Δ*ppiA* Δ*ppiD* Δ*fkpA* Δ*fklB* Δ*surA*) deletion mutants were analyzed by SDS-PAGE and Coomassie blue staining. Indeed, the *surA* single and quintuple mutants showed decreased amounts of proteins in their outer membrane in comparison to the parental bacteria (Supplementary Figure S2A). This defect could be complemented by transforming these *surA* mutants with a low-copy plasmid expressing a wild type copy of *surA*

from its native promoter. Strikingly, the SurA$^+$ quadruple mutant lacking the other four periplasmic PPIase genes exhibited the same outer membrane protein levels as the parental bacteria (Supplementary Figure S2A). To identify some of the reduced outer membrane proteins caused by *surA* deletion, protein bands of interest from parental bacteria were excised from the Coomassie blue-stained gel. Preliminary MALDI-TOF analysis of in-gel digested tryptic fragments identified a 18 kDa protein as Pal (Supplementary Figure S2B) and a 36 kDa protein as OmpA (Supplementary Figure S2C).

Y. pseudotuberculosis lacking *surA* are leaky

An alteration in outer membrane protein profiles suggests a compromised outer membrane. To identify if this induced morphological changes in the affected bacteria, we used TEM to visualize bacterial morphology. Parental bacteria and the quadruple mutant (Δ*ppiA* Δ*ppiD* Δ*fkpA* Δ*fklB*) possessed a uniform peripheral exterior suggestive of an intact bacterial envelope (Figure 6A and 6D). In contrast, the *surA* mutants (Δ*surA* or Δ*ppiA* Δ*ppiD* Δ*fkpA* Δ*fklB* Δ*surA*) displayed an irregular surface and leak material into the surrounding milieu (Figure 6B and 6E). These morphological effects were due to the loss of functional *surA* because a normal morphology could be restored by *trans*-complementation with a wild type copy of *surA* (Figure 6C and 6F).

Despite many bacteria being known to release outer membrane blebs or vesicles, this released material is apparently not vesicular in nature because the structures lack any obvious membranous form (data not shown). To identify the nature of this liberated cellular material, cell-free bacterial supernatants were subjected to ultracentrifugation and the pelleted material collected. No obvious differences in protein content in these samples were observed (data not shown). However, following proteinase K digestion to remove protein material, SDS-PAGE and silver staining analysis revealed an enrichment of released LPS from the quintuple Δ*ppiA* Δ*ppiD* Δ*fkpA* Δ*fklB* Δ*surA* mutant, and to a lesser extent the single Δ*surA* mutant (Supplementary Figure S3A). This was not observed from all other strains examined, including the *trans*-complemented *surA* mutants (Supplementary Figure S3A). Moreover, LPS discharge from the *surA* mutants seems to reflect a defect in LPS assembly control because total cellular LPS levels isolated from whole bacteria were indistinguishable irrespective of the strain background (Supplementary Figure S3B). It is currently unknown whether this putative LPS assembly defect is a direct or indirect consequence of SurA depletion.

Deletion of *surA* confers altered fatty acid and phospholipid composition

Given that *Y. pseudotuberculosis* lacking *surA* have reduced outer membrane protein content and possesses defects in LPS assembly, we undertook a comparative survey of the membrane fatty acid (FA) content of parental and mutant *Y. pseudotuberculosis* grown at ambient (26°C) and elevated temperature (37°C). This raw data can be observed in Supplementary Table S2. At 26°C, unsaturated fatty acids (UFA) were reproducibly produced in abundance by all bacteria (62 to 67.8% of total FA), followed by saturated fatty acids (SFA) (28.8 to 32.6%) and with minor amounts of cyclic fatty acids (CFA) (3.3 to 5.5%) (Figure 7A, upper panel). At 37°C, production of UFA was dramatically curtailed to between 36.7 and 48% of total FA. Interestingly, both *surA* mutants reproducibly produced more UFAs regardless of growth temperature. Consistent with earlier reports [24, 25], CFA production increased dramatically at 37°C (between 12.8 to 23%; Figure 7A, lower panel). However, this effect was noticeably diminished in the *surA* mutants. The ratio of CFA produced by *Y. pseudotuberculosis* at 37°C compared to 26°C was therefore higher for SurA$^+$ bacteria than for SurA$^-$ bacteria (Figure 7B).

On the other hand, the SFA ratios and the UFA ratios remained steady in all bacteria independent of growth temperature (Figure 7B).

A partial analysis of individual phospholipid content within whole bacteria was also determined (Supplementary Table S3). At 26°C, phosphatidylethanolamine (PE) appeared to dominate total phospholipid content (accounting for 79.2 to 81.5%), with phosphatidylglycerol (PG; 8.7 to 11.9%) and cardiolipin (CL; 7.6 to 10.4%) far less prolific (Supplementary Figure S4A). Elevated temperature did not dramatically impact on the relative levels of PE or PG being produced by any of the bacteria (Supplementary Figure S4A). On the other hand, bacteria lacking *surA* or SurA+ bacteria deficient in all four other periplasmic PPIases increased the proportion of CL and other minor phospholipids at elevated temperature (Supplementary Figure S4B). As anticipated from the total cellular FA profiles (see Supplementary Table S2 and Figure 7), the makeup of individual phospholipids at ambient temperature was dominated by UFA, but the relative levels of UFA and SFA equilibrated in bacteria grown at 37°C (Supplementary Figure S4C). In addition, the *surA* mutants routinely produced phospholipids composed of modestly higher levels of UFA irrespective of growth temperature (Supplementary Figure S4C). Thus, subtle alterations in fatty acid and lipid content occur in *Y. pseudotuberculosis* lacking *surA*, but generally not when lacking all four other periplasmic PPIases.

Yersinia lacking _surA_ are susceptible to diverse antimicrobials

Given the obvious perturbations in the bacterial envelope of *Yersinia surA* mutants, we investigated if the envelope barrier function was compromised in these strains. All single mutants, the quadruple mutant and the quintuple mutant were subjected to growth in the presence of antibiotics (vancomycin, bacitracin), bile salts (a major component contained within YSA) and detergents (SDS, sodium deoxycholate). Quite expectedly, the two *Y. pseudotuberculosis* mutants lacking *surA* were more susceptible to these antimicrobials compared to any other strain (Supplementary Figure S5A and S5B). Envelope barrier function was restored to *surA* mutants by ectopic expression of a wild-type copy of *surA*. Not surprisingly, the *surA* mutants were modestly less able to maintain long-term viability during extended culturing (Supplementary Figure S5C). Remarkably, none of these conditions impeded growth of the $SurA^+$ quadruple mutant. Overall, this reflects a strict requirement of SurA for maintenance of envelope integrity in pathogenic *Y. pseudotuberculosis*.

PPIases other than SurA are important for _Yersinia_ survival during exposure to ROIs

A confounding aspect of this study has been the lack of discernable phenotypes for the quadruple Δ*ppiA* Δ*ppiD* Δ*fkpA* Δ*fklB* mutant that still encodes an intact *surA* allele. Since it is unlikely that *Y. pseudotuberculosis* would retain proteins in the absence of any important function, we assessed the sensitivity of our mutants to hydrogen peroxide, the super-oxide generating compound menadione and acid stress. These are relevant stresses that must be counteracted by *Y. pseudotuberculosis* in order to survive during an intracellular lifecycle inside an infected host [26]. Decisively, exposure to hydrogen peroxide resulted in significant (*, $p < 0.05$) growth defects of the quadruple mutant, although to a lesser degree than *Y. pseudotuberculosis* lacking *surA* (Supplementary Figure S6). This result agrees with the recent finding that a Par10-negative *E. coli* strain was also more sensitive toward hydrogen peroxide than was the wild-type strain [27]. Significantly, the presence of hydrogen peroxide is the first *in vitro* growth condition in which the quadruple mutant displayed a reduced tolerance to stress. Interestingly however, this mutant did grow to the same level as parental bacteria in the presence of 200 μM menadione,

whereas *surA* mutants still displayed a significant growth defect (Supplementary Figure S6). These differential sensitivities of bacteria to hydrogen peroxide and menadione is not uncommon [28], possibly because the oxidative stress pathways for the superoxide and hydrogen peroxide sensing are independently regulated by SoxRS and OxyR respectively [29].

We also tested the sensitivity of these bacteria to increasingly acidic conditions. Despite pH 5.0 being bacteriostatic and pH 3.0 being bacteriocidal for *Y. pseudotuberculosis*, we could not detect any differences between parental and mutant bacteria at either growth condition (data not shown). Thus unlike their protective effect against the action of ROIs, no periplasmic PPIase appeared to confer resistance to *Y. pseudotuberculosis* during growth in low pH.

PPIases and *Y. pseudotuberculosis* virulence

The quadruple mutant (Δ*ppiA* Δ*ppiD* Δ*fkpA* Δ*fklB*) exhibited an *in vitro* growth defect in the presence of hydrogen peroxide – an antimicrobial compound commonly associated with activation of host immune cells during microbial infection. In light of this, we were keen to address if this mutant was attenuated during *in vivo* infections of female BALB/c mice. We scored attenuation on the basis of ID_{50} as determined by the extent of visible symptoms of infection, such as ruffled fur, weight loss, listlessness and diarrhoea. Mice infected with high doses (10^6 and 10^5) of parental *Y. pseudotuberculosis* were terminally infected within 4 and 8 days, respectively (Figure 8A). With these trends an ID_{50} of 2.0×10^4 could be calculated for the parental strain. On the other hand, mice infected with the single *surA* mutant, the quadruple PPIase mutant or the quintuple PPIase mutant were mostly symptom free (Figure 8A). This suggested that ID_{50} measurements for these mutant bacteria were in excess of 3.0×10^6, 3.2×10^6 and 2.8×10^6, respectively; a 100-fold attenuation compared to the parent. Interestingly, we did observe a small degree of weight loss (Figure 8B) and ruffled fur (data not shown) in those mice infected with higher doses of the quadruple PPIase mutant. This was not evident in infections with *surA* mutant bacteria. We therefore conclude from these *in vivo* mouse infection studies that full *Y. pseudotuberculosis* pathogenicity does depend on a functioning collection of several periplasmic PPIases, and not solely on active SurA. Hence, one or more of the other periplasmic PPIases possess an important role in *Y. pseudotuberculosis* survival in a particular niche inside the infected host. We therefore interpret the two-log reduction in ID_{50} observed for $SurA^+$ and $SurA^-$ bacteria to reflect SurA-dependent and SurA-independent modes of virulence attenuation.

DISCUSSION

In comparison to SurA [6], it has proven much more difficult to establish the physiological roles of the other periplasmic PPIases. FkpA is a PPIase with chaperone activity [23, 30] that might cooperate with SurA in the starvation stress response [31]. PpiD was originally described as a multicopy suppressor of *surA* [22] with recent reports also providing evidence of a periplasmic chaperone role [32, 33]. On the other hand, FklB and PpiA functions still remain obscure. We used two types of functional assay to study a quadruple Δ*ppiA* Δ*ppiD* Δ*fkpA* Δ*fklB* deletion mutant still containing functional SurA, a single Δ*surA* mutant, and a quintuple Δ*ppiA* Δ*ppiD* Δ*fkpA* Δ*fklB* Δ*surA* deletion mutant lacking all five extracytoplasmic PPIases; those that broadly examined bacterial envelope integrity and those that scrutinize survival in the host. Only in the absence of SurA could we detect obvious defects in *Yersinia* envelope integrity. Yet, like the *surA* mutants, $SurA^+$ bacteria lacking all other periplasmic PPIases were sensitive to conditions that mimicked the vacuolar environment inside an activated host immune cell (such as

elevated hydrogen peroxide). Indeed, PPIases could have an important role during oxidative stress [27]. To this end, all three mutant bacteria were also highly attenuated in a mouse-infection model. These data imply that SurA cannot compensate for the collective loss of PpiA, PpiD, FkpA and FklB when *Y. pseudotuberculosis* enters a mammalian host, but it can during routine *in vitro* culturing. SurA is therefore generally required for bacterial survival during both *in vitro* and *in vivo* conditions, while all other periplasmic PPIases are required during growth in a particular ecological niche; most notably during mammalian infections. The mechanistic basis for these attenuating effects remain obscure; to define the *in vivo* roles for the PpiA, PpiD, FkpA and FklB PPIases during *Y. pseudotuberculosis* pathogenesis is a goal for the future. We anticipate that their roles in promoting pathogenicity are likely to be indirect. Perhaps in the absence of PpiA, PpiD, FkpA or FklB, one or more of their native substrates that are also important for *Yersinia* survival *in vivo,* fail to assemble in the outer membrane.

Having purified four of the five PPIases from *Y. pseudotuberculosis,* specific antibodies were generated to enable a definitive investigation into sub-cellular localization of SurA, PpiD, FkpA and FklB. While the localizations of SurA and FkpA in the periplasmic fraction and PpiD in the inner membrane are consistent with observations from *E. coli* [22, 23], we identified FklB to be predominately associated with the inner membrane, not the periplasm as previously reported for *E. coli* [5]. These two assays were performed differently, which may account for this discrepancy. On the other hand, FklB was recently seen to interact with inner membrane-associated thioredoxin [34]. Perhaps this is a transient interaction that could occasionally cause FklB to locate to the inner membrane. Further work is needed to resolve this inconsistency. Nonetheless, the extracytoplasmic compartmentalization of these PPIases in *Yersinia* ideally positions them for interactions with early protein folding intermediates in the periplasm.

SurA is the subject of intensive research (reviewed in [6]). It is critical for periplasmic quality control, particularly when the bacteria are faced with optimizing growth and survival in adverse environmental conditions. Although long regarded as a PPIase enzyme, evidence now supports a primary role of SurA as a chaperone assisting in the transport and assembly of a subset of outer membrane proteins [35, 36]. In fact, the requirement for SurA PPIase-independent chaperone activity supersedes the need for its PPIase activity [35]. This could explain why we observed such low *in vitro* PPIase activity from SurA derived from *Y. pseudotuberculosis*, since the two Parvulin domains and the associated PPIase activity may only play a secondary role in SurA function. However, a role for the SurA Parvulin domains in protein folding *in vivo* during growth of the organism in some defined ecological niche should not be discounted. We intend to generate site-directed *surA* mutants to pinpoint whether the physiological defects in our existing full-length *surA* mutants are due to loss of SurA PPIase activity or chaperone activity.

Target substrates of SurA are thought to possess a W-X-W motif (where W is an aromatic amino acid) that is a characteristic of β-barrel proteins [37-39]. In our preliminary search for potential SurA targets in *Yersinia*, we identified a significant reduction of OmpA and the Pal lipoprotein in outer membrane fractions. OmpA, containing a singular W-X-W motif, constitutes a major component of the bacterial outer membrane and therefore possesses important pleiotropic functions [40]. OmpA levels are noticeably lessened in *E. coli* mutants lacking *surA* [17, 41] and SurA can interact with OmpA peptides [38]. However, it is unclear whether this interaction is a requirement for OmpA assembly in the outer membrane because *ompA* transcription is also diminished in *surA* mutants [42] and another periplasmic chaperone, Skp, is already implicated in OmpA assembly [43]. The outer membrane Pal lipoprotein also performs essential functions in the bacterial envelope [44]. As an integral component of the Tol-Pal

system, it bridges the inner and outer membranes with the intervening peptidoglycan layer. Formation of these molecular bridges is dependent on the proton motive force and is necessary for cell division, solute transport across the inner membrane and the preservation of outer membrane integrity. Pal transport to the outer membrane is dependent on the Lol pathway [45]. Since Pal is not considered a true substrate of SurA [42], loss of *surA* may decrease Lol-dependent transport efficiency, perhaps as a result of alterations in membrane lipid content (discussed below) [46]. To define the true molecular targets of SurA in *Y. pseudotuberculosis* might well identify novel virulence factors essential for *Yersinia* survival during host infections.

To optimize growth in varied environments, bacteria actively regulate fatty acid composition to modulate lipid content and distribution in order to maintain membrane structure and function [47-49]. Clinically important bacteria, such as the *Yersiniae*, also alter lipid content as a means to evade the hostile host innate immune response [50, 51]. Significantly, alterations in fatty acid and lipid content specifically occurred in *Y. pseudotuberculosis* lacking *surA*. Presumably, this limits the adaptiveness of *surA* mutants to extracytoplasmic stress and the ability to instigate outer membrane repair, especially at elevated temperatures; a poorer survival outcome would result. SurA influence on fatty acid and lipid content could be direct or indirect, affecting *de novo* fatty acid or lipid biosynthesis, transport and assembly and/or modification of existing fatty acids or lipid structures. A simple explanation is that *surA* depletion indirectly stimulates an increased production of key fatty acids or lipids to compensate for the compromised envelope. This precedent exists given that chronic extracytoplasmic stress responsiveness affects LPS biogenesis [52] and deletion of *surA* constitutes a strong extracytoplasmic stress stimulant in *E. coli* [17]. It is also in balance with our observations that *Yersinia surA* mutants release significant amounts of LPS into the surrounding milieu. In view of this, it would be interesting to examined σ^E or Cpx pathway activity in *Yersinia* lacking *surA*. Knowing this may benefit our future understanding of the molecular connection between SurA and lipid biosynthesis/assembly in the *Yersinia* envelope.

AUTHOR CONTRIBUTION

Ikenna Obi, Roland Nordfelth and Matthew Francis designed, performed and analysed experiments. Ikenna Obi and Matthew Francis wrote the paper. All authors read and approved the final paper.

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FIGURE LEGENDS

Figure 1 Periplasmic PPIases of *Y. pseudotuberculosis* YPIII
Schematic representation of the genetic organization of gene loci of *Y. pseudotuberculosis* periplasmic PPIases (**A**). *surA* (NCBI annotation YPK_3571), *ppiA* (YPK_0242) and *fkpA* (YPK_0270) are all coded on the sense strand at genome positions 3935985 – 3937289, 284007 – 284576 and 309956 – 310756, while *ppiD* (YPK_3230) and *fklB* (YPK_3778) are coded on the complementary strand at genome positions 3531083 – 3532969 and 4168658 – 4169278, respectively. Symbols denote genes encoding helix-hairpin-helix repeat-containing competence protein ComEA (§), transcriptional regulator subunit beta (*), glycoside hydrolase family protein (**), putative NPT hydrolase (***), SlyX (+), YheO domain-containing protein (++), iron-sulfur cluster repair di-iron protein (#) and opacity-associated protein A (##). Predicted gene names are given in parenthesis or denoted by a symbol to reflect function. Amino acid sequence identity of periplasmic PPIases from *Y. enterocolitica* 8081, *Y. pestis* CO92, *E. coli* K12 MG1655 and *Y. pseudotuberculosis* YPIII (**B**). Amino acid sequences were aligned using the BLAST algorithm. NCBI annotation is provided in square brackets and the numbers in round brackets represents the length of each protein in amino acids. Domain organization of periplasmic PPIases from *Y. pseudotuberculosis* (**C**). The predicted molecular weight for each protein is provided in parentheses.

Figure 2 Purification of *Y. pseudotuberculosis* periplasmic PPIases
The purified recombinant proteins over-produced in *E. coli* BL21 were boiled for 5 min in SDS sample buffer and separated on 12% SDS polyacrylamide gels. Proteins were stained with Coomassie Blue. Approximate molecular weights of the individual protein bands in the PageRuler Prestained Protein ladder (Fermentas Life Sciences) are provided to the left of the panel. Adjacent lanes are purified recombinant SurA, PpiD, FkpA and FklB, respectively.

Figure 3 PPIase activity of recombinant SurA, PpiD, FkpA and FklB
Progress curves for the *cis/trans* isomerization of the chromogenic peptide Suc-Ala-Leu-Pro-Phe-pNa (**A**). α-chymotrypsin-mediated hydrolysis of Suc-Ala-Leu-Pro-Phe-pNa was measured in the absence or presence of 0.1 μM recombinant SurA, PpiD, FkpA, FklB or CpxR (protein negative control) at 10 °C for 3 minutes. PPIase activity of SurA, FkpA and FklB is concentration dependent (**B**). Various concentrations of the recombinant proteins were assayed for PPIase activity and the rate constants were calculated by fitting the absorbance data to a first-order exponential rise function. All data-points were an average of three independent measurements (± standard deviation) and were highly reproducible.

Figure 4 Inhibition of PPIase activity with immunosuppressive drugs
Inhibition assay was measured with 0.1 μM SurA (**A**), FkpA (**B**) or FklB (**C**) in 50mM Tris-HCl, pH 8 at 10 °C using Suc-Ala-Leu-Pro-Phe-pNa as substrate in the presence of different concentrations of the immunosuppressants CsA, FK506 and rampamycin prepared in ethanol/water (1:1, v/v). Remaining activity was calculated using the rate constants in the presence of the inhibitors. Note that the final concentration of ethanol in the assay mixture was 1 %, but this had negligible effect on PPIase activity as determined by control assays in the presence of ethanol alone (data not shown). All data-points were an average of three independent measurements (± standard deviation) and were highly reproducible.

Figure 5 Sub-cellular localization of SurA, PpiD, FkpA and FklB in *Y. pseudotuberculosis*
An overnight culture of *Y. pseudotuberculosis* was sub-cultured in fresh LB broth. After 2 h, the bacteria were harvested and whole cell-lysate was separated into outer membrane, periplasmic, inner membrane and cytoplasmic fractions. TolC, MBP, FtsH and H-NS were used as outer membrane, periplasmic, inner membrane and cytoplasmic markers to control the purity of the fractionation, respectively. Each cellular fraction was then probed with anti-SurA, anti-PpiD, anti-FkpA and anti-FklB antibodies.

Figure 6 Transmission electron microscopy of PPIase mutants of *Y. pseudotuberculosis*
Cultures were grown on LB agar at 26 °C prior to the TEM analysis of the parental strain (**A**), *surA* single mutant (Δ*surA*) (**B**), *surA* single mutant (Δ*surA*) *trans*-complemented with *surA* (**C**), quadruple mutant (Δ*ppiA* Δ*ppiD* Δ*fkpA* Δ*fklB*) (**D**), quintuple mutant (Δ*ppiA* Δ*ppiD* Δ*fkpA* Δ*fklB* Δ*surA*) (**E**), quintuple mutant (Δ*ppiA* Δ*ppiD* Δ*fkpA* Δ*fklB* Δ*surA*) *trans*-complemented with *surA* (**F**). Arrows indicate the abundance of material released into the extracellular milieu by *Yersinia* mutants lacking *surA.* Scale bar = 0.5 μm.

Figure 7 Comparative analysis of the fatty acid levels in isogenic strains of *Y. pseudotuberculosis* lacking periplasmic PPIases
Relative fatty acid levels were calculated from the free mg fatty acid/100 g total lipid samples (see Supplementary Table S2) extracted from exponentially grown parent, *surA* single mutant (Δ*surA*), quadruple mutant (Δ*ppiA* Δ*ppiD* Δ*fkpA* Δ*fklB*) and quintuple mutant (Δ*ppiA* Δ*ppiD* Δ*fkpA* Δ*fklB* Δ*surA*) bacteria. Raw data is represented as the percentage (%) of saturated (SFA), unsaturated (UFA) and cyclic (CFA) fatty acids from bacteria grown at 26°C and 37°C (**A**) and the ratio of SFA, UFA and CFA content at 37°C compared to 26°C (**B**). Shown is the average of three highly reproducible measurements (± standard deviation).

Figure 8 PPIases are required for virulence in mice
Dilutions of *Y. pseudotuberculosis* parent and mutant bacteria were prepared from washed pellets that were serially diluted to 10^7, 10^6 and 10^5 CFU/ml in PBS. Mice were then injected intraperitoneally with 0.1 ml to give final inocula of approximately 10^6, 10^5 and 10^4 bacteria per mouse (5 mice for each strain and dilution). Mice were observed for 14 days post-injection for survival (**A**) and alterations in their body weight (**B**). In **A**, only mice infected with dilutions of parental bacteria show any visible signs of infection. Hence much of the data reflects healthy symptom-free mice that are concealed by the bold solid grey line stylised with a filled square symbol.

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype	Source or reference
Strain		
E. coli		
DH5	F$^-$ *recA*1, *endA*1, h*sdR*17, *supE*44, *thi*-1, *gyrA*96, *relA*1	Vicky Shingler
BL21	F$^-$ *dcm ompT hsd*S(r_B- m_B-) *gal* (*mal*B^+)$_{K-12}$(λ^S)	Novagen
S17-1λ*pir*	*rec*A, *thi*, *pro*, *hsdR*$^-$*M*$^+$, Sm^R, <RP4–2-Tc–Mu–Ku–Tn7 >Tp^R	[53]
Y. pseudotuberculosis		
YPIII/pIB102	*yadA*::Tn5 (parent), inactive PhoP, Km^R	Hans Wolf-Watz
YPIII20/pIB102	*ppiA* in-frame deletion of codons 49 to 177, Km^R	This study
YPIII21/pIB102	*surA* in-frame deletion of codons 26 to 411, Km^R	This study
YPIII22/pIB102	*fklB* in-frame deletion of codons 16 to 188, Km^R	This study
YPIII38/pIB102	*fkpA* in-frame deletion of codons 20 to 247, Km^R	This study
YPIII53/pIB102	*ppiD* in-frame deletion of codons 14 to 604, Km^R	This study
YPIII73/pIB102	*ppiA*, *ppiD*, *fklB, fkpA* in-frame deletion, Km^R	This study
YPIII80/pIB102	*surA*, *ppiA*, *ppiD*, *fklB, fkpA* in-frame deletion, Km^R	This study
Plasmid		
pDM4	Suicide mutagenesis plasmid carrying *sacBR*, Cm^R	Debra Milton
pMF658	~465 bp *XhoI*/*XbaI* PCR fragment encoding the full-length *surA* deletion allele in pDM4, Cm^R	This study
pMF653	~435 bp *XbaI*/*XhoI* PCR fragment encoding the full-length *ppiA* deletion allele in pDM4, Cm^R	This study
pMF688	~475 bp *XhoI*/*XbaI* PCR fragment encoding the full-length *ppiD* deletion allele in pDM4, Cm^R	This study
pMF679	~505 bp *XhoI*/*XbaI* PCR fragment encoding the full-length *fkpA* deletion allele in pDM4, Cm^R	This study
pMF655	~530 bp *XhoI*/*XbaI* PCR fragment encoding the full-length *fklB* deletion allele in pDM4, Cm^R	This study
pET30a(+)	Expression vector, Km^R	Novagen
pIR001	~621 bp *NcoI*/*HindIII* PCR fragment of *fklB* in pET30a(+), Km^R	This study
pIR002	~801 bp *NcoI*/*HindIII* PCR fragment of *fkpA* in pET30a(+), Km^R	This study
pIR003	~1305 bp *NcoI*/*HindIII* PCR fragment of *surA* in pET30a(+), Km^R	This study
pIR004	~570 bp *NcoI*/*HindIII* PCR fragment of *ppiA* in pET30a(+), Km^R	This study
pIR012	~1887 bp *NcoI*/*BamHI* PCR fragment of *ppiD* in pET30a(+), Km^R	This study
pWKS30	Low-copy number cloning plasmid, Cb^R	Sidney Kushner
pIR005	~2200 bp *XbaI*/*XhoI* PCR fragment of *surA* with its native promoter in pWKS30, Cb^R	This study

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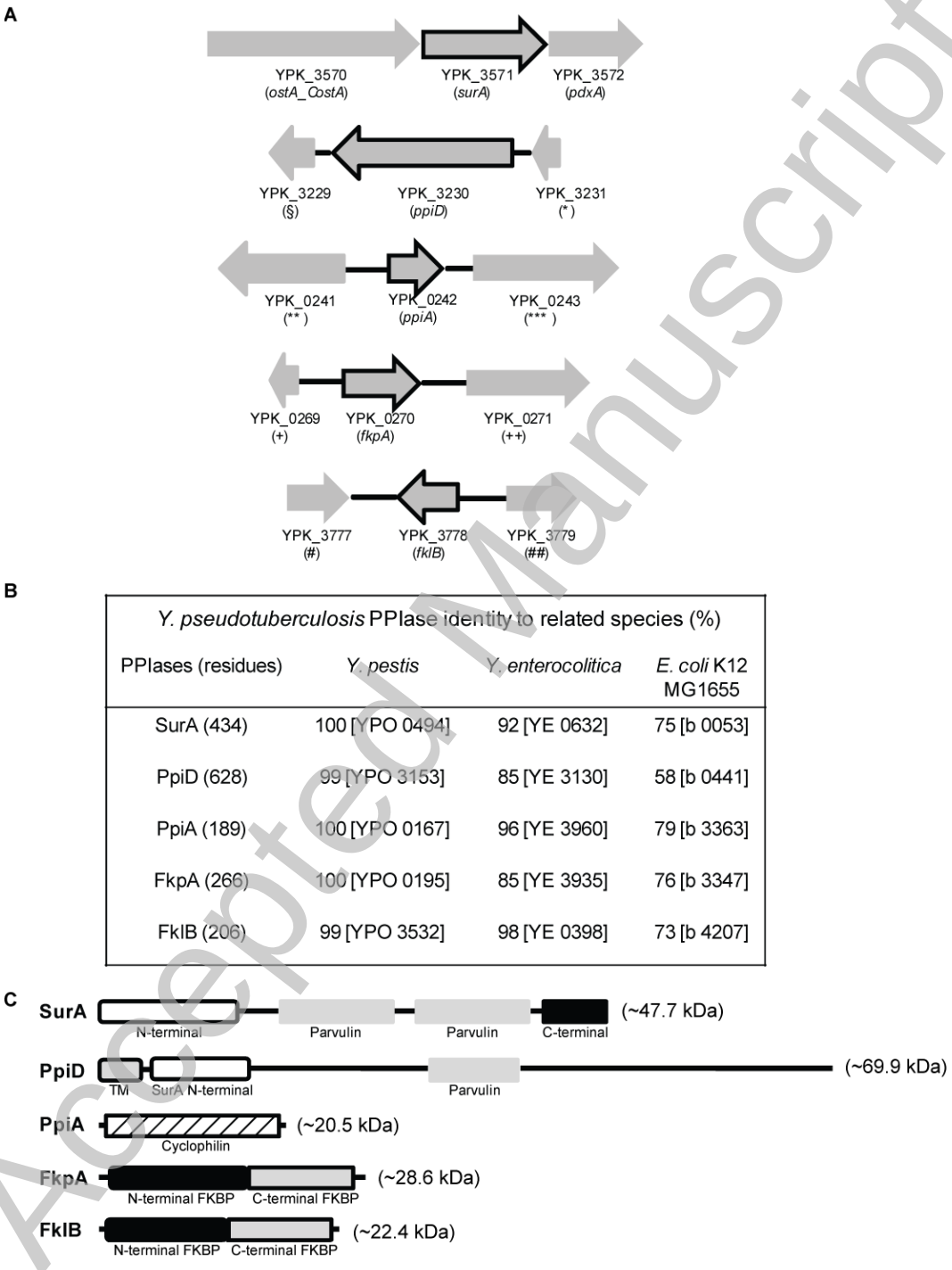
Figure 1: Obi et al., 2011



Y. pseudotuberculosis PPIase identity to related species (%)

PPIases (residues)	*Y. pestis*	*Y. enterocolitica*	*E. coli* K12 MG1655
SurA (434)	100 [YPO 0494]	92 [YE 0632]	75 [b 0053]
PpiD (628)	99 [YPO 3153]	85 [YE 3130]	58 [b 0441]
PpiA (189)	100 [YPO 0167]	96 [YE 3960]	79 [b 3363]
FkpA (266)	100 [YPO 0195]	85 [YE 3935]	76 [b 3347]
FklB (206)	99 [YPO 3532]	98 [YE 0398]	73 [b 4207]

Figure 2: Obi et al., 2011

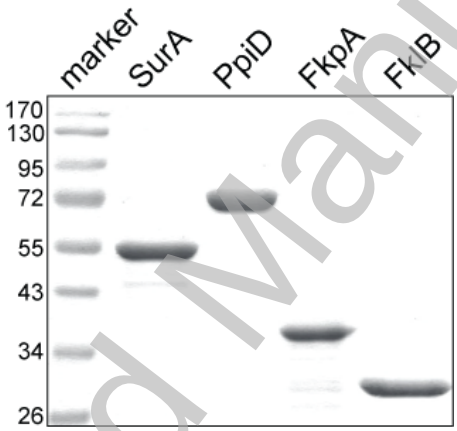


Figure 3: Obi et al., 2011

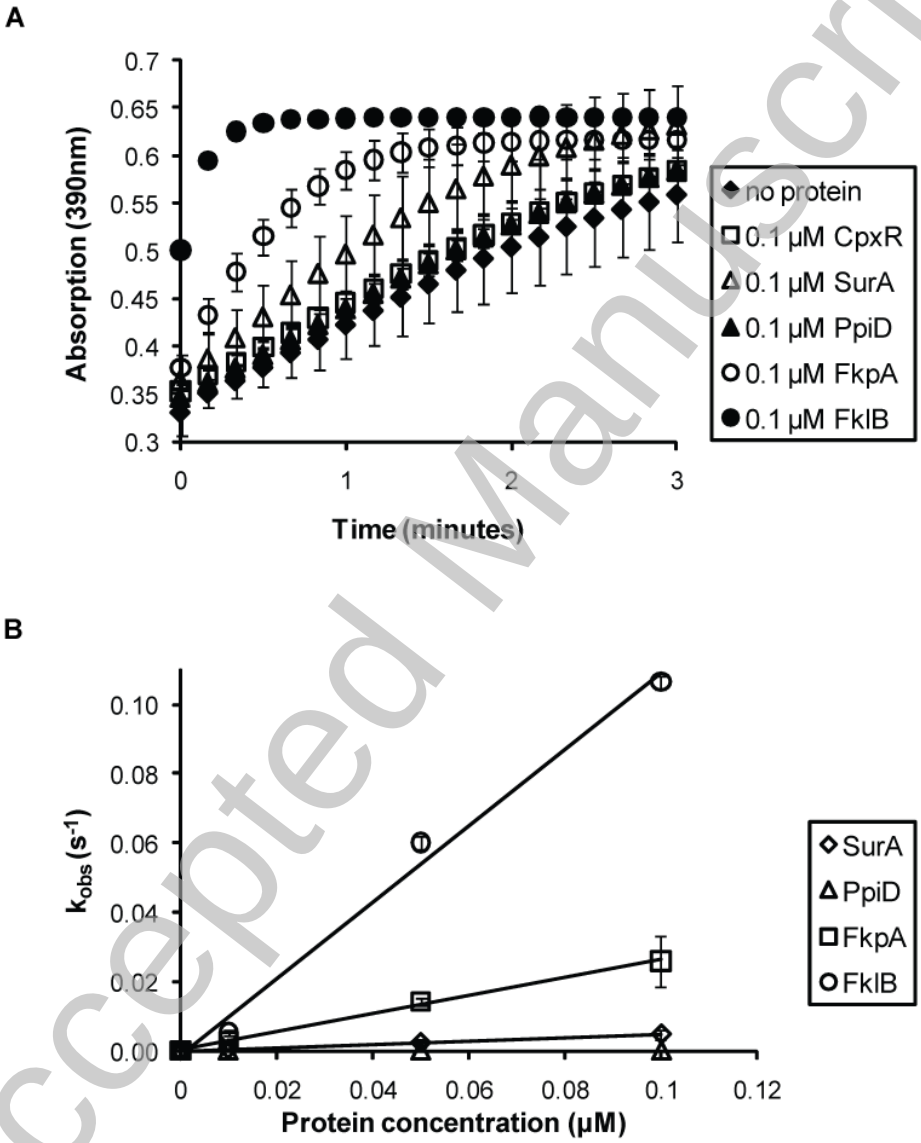
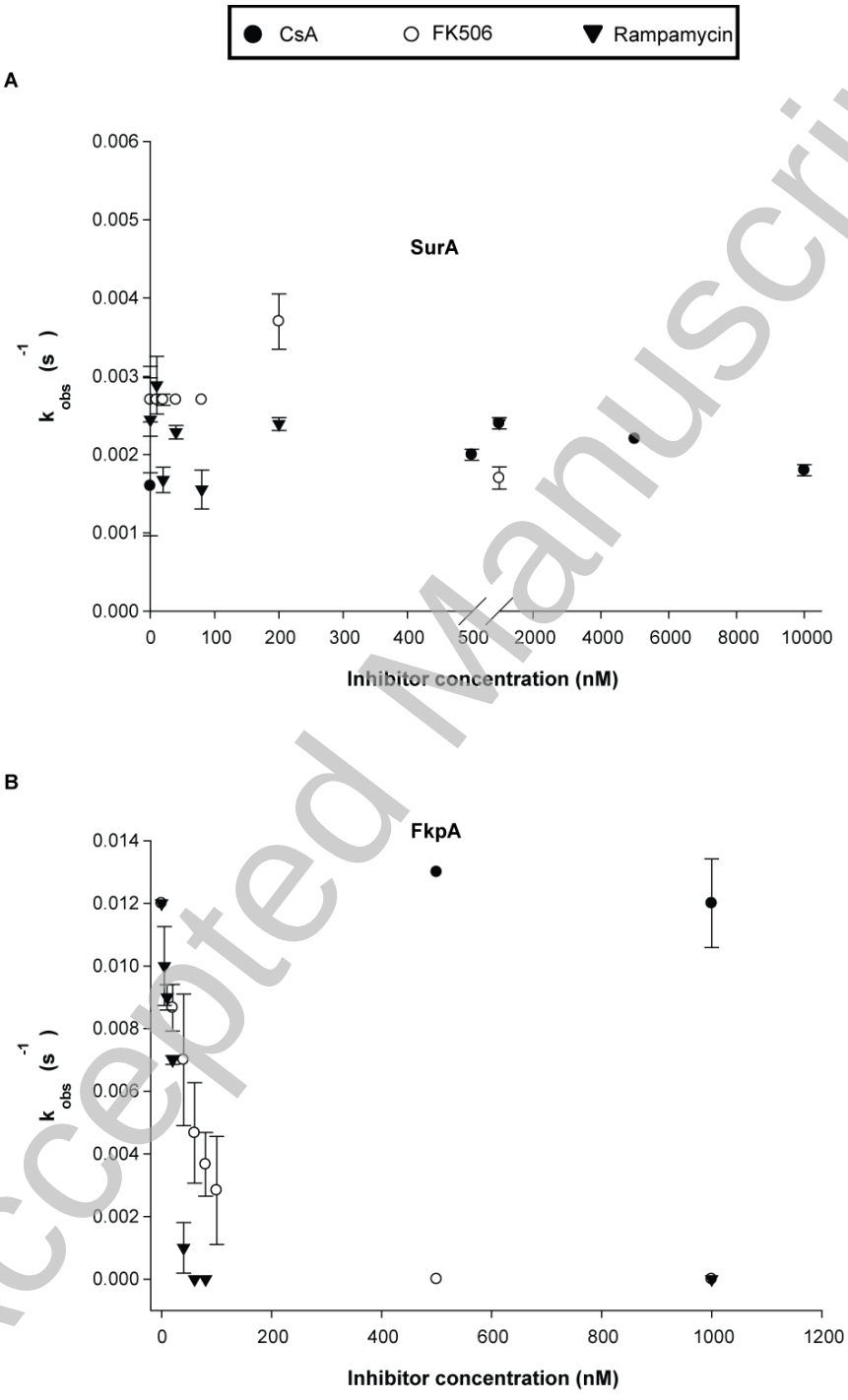


Figure 4: Obi et al., 2011



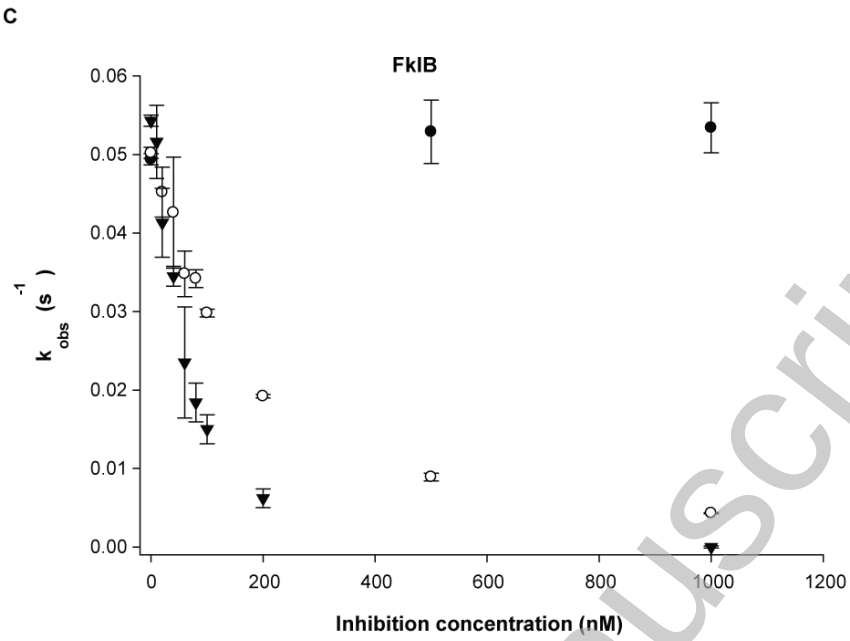


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Figure 5: Obi et al., 2011

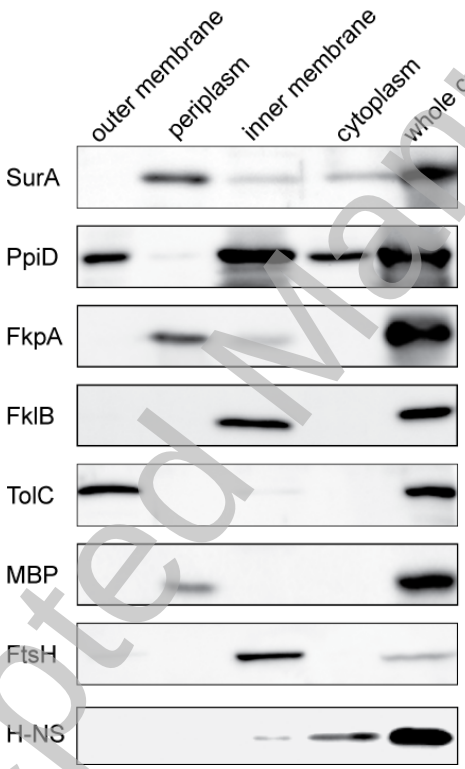

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Figure 6: Obi et al., 2011

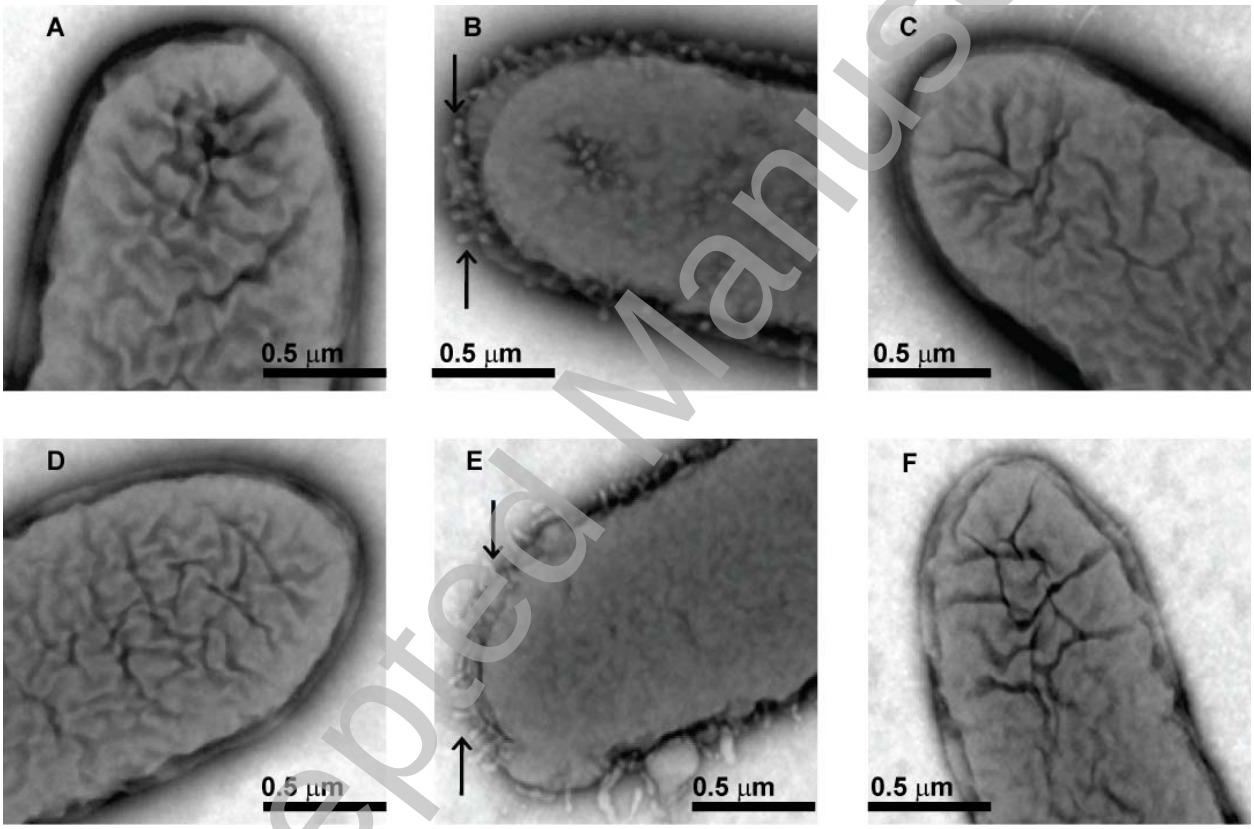

Figure 7: Obi et al., 2011

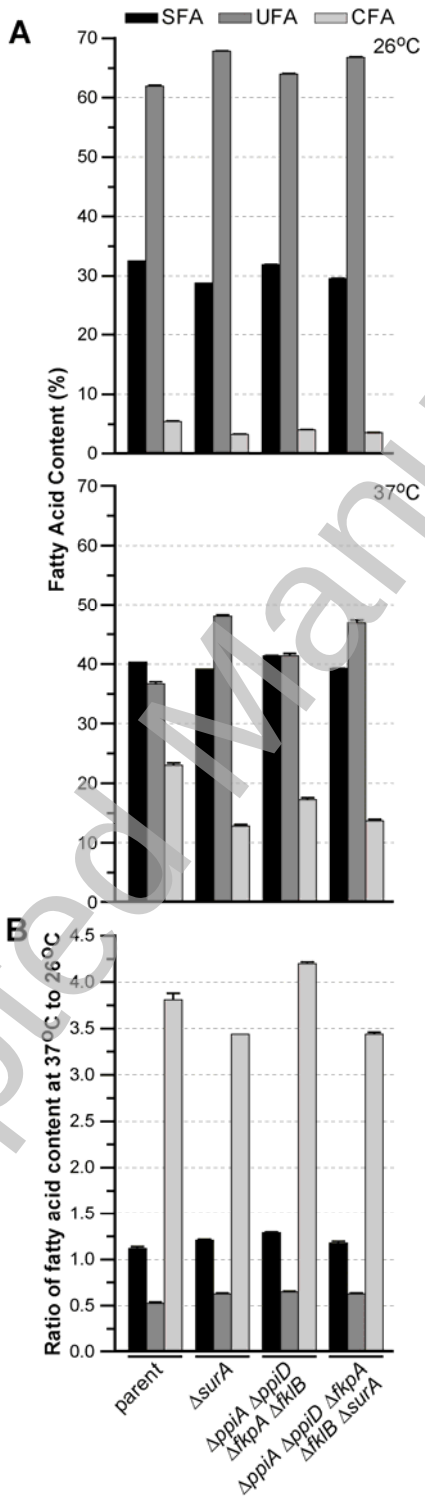

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Figure 8: Obi et al., 2011