Francisella DnaK Inhibits Tissue Nonspecific Alkaline Phosphatase

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Background: Pulmonary Francisella infection resulted in reduction of plasma alkaline phosphatase activity.

Results: Francisella heat shock protein DnaK binds to alkaline phosphatase reducing enzymatic activity.

Conclusion: A Francisella protein component responsible for alkaline phosphatase inhibition was identified.

Significance: We present a novel mechanism used by a bacterial pathogen to evade the host’s defense.

SUMMARY

Following pulmonary infection with Francisella tularensis, we observed an unexpected but significant reduction of alkaline phosphatase, an enzyme normally up-regulated following inflammation. However, no reduction was observed in mice infected with a closely related Gram negative pneumonic organism (Klebsiella pneumonia) suggesting the inhibition may be Francisella specific. In similar fashion to in vivo observations, addition of Francisella lysate to exogenous alkaline phosphatase (tissue nonspecific isozyme) is inhibitory. Partial purification and subsequent proteomic analysis indicated the inhibitory factor to be the heat shock protein DnaK. Incubation with increasing amounts of anti-DnaK antibody reduced the inhibitory effect in a dose-dependent manner. Furthermore, DnaK contains an adenosine triphosphate binding domain at its N-terminus, and addition of adenosine triphosphate enhances dissociation of DnaK with its target protein, e.g., alkaline phosphatase. Addition of adenosine triphosphate resulted in decreased DnaK co-immunoprecipitated with alkaline phosphatase as well as reduction of Francisella-mediated alkaline phosphatase inhibition further supporting the binding of Francisella DnaK to alkaline phosphatase. Release of DnaK via secretion and/or bacterial cell lysis into the extracellular milieu and inhibition of plasma alkaline phosphatase could promote an orchestrated, inflammatory response advantageous to Francisella.

Francisella tularensis is a facultative intracellular, Gram negative bacterium that causes the zoonotic disease pulmonary tularemia (1,2). Several F. tularensis species and subspecies are recognized including (i) F. tularensis subsp. tularensis (type A), (ii) F. tularensis subsp. holarctica (type B), (iii) F. tularensis subsp. mediasiatica, and (iv) F.
novicida (1). While type A and B strains are the most relevant in terms of human disease, F. novicida and the live vaccine strain F. tularensis LVS (derived from holarctica) are attenuated in humans while retaining virulence in mice (3-5). F. novicida exhibits > 95% genetic homology and shares biochemical features with type A (6). We have previously reported that in a murine pneumonic tularemia model, F. novicida rapidly disseminated from the challenge site (lungs) to liver with a progressive increase in bacterial load by 72 h (7). Liver damage resulting from pulmonary F. novicida infection was assessed by analyzing liver function enzymes in plasma and a marked decrease in total alkaline phosphatase (AP) activity as early as 48 h after pulmonary challenge was observed. This observation of decreased AP was unexpected since most reported pathogen infections give rise to increased AP activity.

Alkaline phosphatase [(AP), orthophosphoric monoesterphophohydrolase, alkaline optimum, EC 3.1.3.1] is responsible for removing phosphate groups from a wide variety of molecules. In mice, there are four genes coding for AP: intestinal, placental, germ cell, and tissue nonspecific (TNAP). The latter form is post-translationally modified to differentiate the bone, liver, and kidney isoforms. There is growing evidence to suggest that AP may play an important role in host defense. Within primary sites of infection, such as the lung, AP is expressed at a high level and may be produced in pulmonary surfactant particles by type-II pneumocytes (8). Alkaline phosphatase has been shown to detoxify Gram-negative LPS by the removal of terminal phosphate groups (9-11), and AP synthesized by hepatocytes has been reported to play a protective role during liver damage by the neutralization of endotoxin (12,13). However, the LPS of F. tularensis exhibits an unusual lipid A structure that does not contain exposed phosphate groups and generally exhibits low endotoxicity (14,15). Moreover, in our studies, purified LPS from F. novicida and F. tularensis LVS demonstrated no measurable effect on host AP activity, indicating that LPS was not involved further suggesting involvement of other bacterial factors.

In this study, F. novicida lysate protein was subjected to anion exchange chromatography and electrophoretic separation. Using an in vitro assay, inhibition of AP was determined. We provide evidence that heat shock protein DnaK of F. novicida binds to AP reducing enzymatic activity. This is the first report of such a novel mechanism used by a pathogen to evade the host’s defense.

EXPERIMENTAL PROCEDURES

Bacterial strains-Francisella novicida strain U112, F. tularensis subsp. tularensis (type A, SCHU S4 strain), F. tularensis subsp. holarctica strains (type B, OR96-0246 and LVS, lot 703-0303-016), Klebsiella pneumoniae (KPPR1 strain) (16), and Salmonella typhimurium (ATCC, strain 14028) were inoculated in trypticase soy broth (TSB) supplemented with 0.1 % (w/v) L-cysteine hydrochloride, 0.025 % (w/v) sodium pyruvate, 0.025 % (w/v) sodium metabisulphite, and 0.025 % (w/v) FeSO4. After reaching stationary phase, cells were harvested by centrifugation, and stored at -80 °C until used.

Preparation of Plasma-Female BALB/c mice (5-8 weeks) were obtained from the National Cancer Institute (Frederick, MD). All animal care and experimental procedures were performed in compliance with the Institutional Animal Care and Use Committee (IACUC) guidelines. Mice were challenged intranasally (i.n.) with 100 cfu of either F. tularensis type A (LD50 < 10 cfu) or F. tularensis type B (LD50 = 10 cfu) in 25 µl phosphate buffered saline (PBS) or with 400 cfu F. novicida (LD50 = 10 cfu), LVS (LD50 = 2800 cfu), or K. pneumoniae (LD50 < 100 cfu). Mice were bled at 0, 24, 48, and 72 h post challenge, and plasma prepared using plasma collection tubes containing lithium and heparin sulfate (Fisher Scientific, NJ). Respective plasma samples were centrifuged for 5 min at 5000 rpm, and aliquots frozen at -20 °C until used.

Plasma biochemical assays-Plasma albumin content, as well as alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (AP) levels were measured...
Running title: Alkaline phosphatase inhibition and Francisella survival

at the University of Texas Health Science Center at San Antonio using an Olympus AU640e Chemistry Immuno Analyzer (Olympus, Center Valley, PA). Plasma from infected mice also was analyzed for AP activity (nmol or pmol/min/µl) in 96 well microplates by measuring the rate of hydrolysis of paranitrophenyl phosphate (PNPP) (Sigma-Aldrich, St. Louis, MO) or 4-methylumbelliferyl phosphate (4-MUP) (Sigma-Aldrich) as previously described (17,18). Briefly, plasma samples (10 µl) were added to 190 µl of a substrate solution containing 1.9 mM PNPP dissolved in AP buffer (0.1 M glycine buffer, pH 7.4 containing 1 mM MgCl$_2$ and 1 mM ZnCl$_2$). Microplates were incubated at 37 °C, and substrate hydrolysis monitored spectrophotometrically at 410 nm every 10 min for 1 h using a µQuant Microplate Spectrophotometer (Biotek, Winooski, VT). For fluorometric analyses, plasma samples (10 µl, 1:10 diluent) were added to 190 µl of a substrate solution containing 5 mM 4-MUP dissolved in AP buffer. Microplates were incubated at 37 °C with moderate shaking, and the hydrolysis of substrate was monitored fluorometrically at 360 nm (excitation) and 465 nm (emission) every 10 min for 1 h using a Synergy HT Multidetection Plate Reader (Biotek). Quantitation of substrate hydrolysis was determined using either a linear paranitrophenol (PNP; 0-60 nmol) or 4-methylumbelliferyl (4-MU; 0-600 pmol) standard curve generated under identical assay conditions but in the absence of PNPP or 4-MUP, respectively.

**Detection of AP by zymogram analysis**

Samples were loaded onto 4-15 % gradient polyacrylamide gels (BioRad Laboratories, Carlsband, CA), and run under native conditions at 180V for 2 h after which the gel was washed 3 times with 10 mM Tris buffer, pH 7.4. Following washing, gels were incubated with substrate solution (5 mM 4-MUP dissolved in 25 ml of AP buffer) for 15 min. The reaction was stopped by addition of 25 ml 0.10 M NH$_4$OH, pH 10.4, and protein bands associated with hydrolyzed 4-MUP, i.e., 4-MU were observed and photographed under UV light.

**Bacterial lysate preparation**

*F. novicida*, *K. pneumoniae*, and *S. typhimurium* were grown as described earlier and cells harvested by centrifugation. Following suspension in 5 ml chilled 10 mM Tris buffer, pH 7.4, cells were ruptured using a French Pressure Cell Press (American Instrument Co., Silver Spring, MD). Ruptured cells were centrifuged at 30,000 x g for 30 min, and lysate supernatant material was stored at -80° C until used. Only minimal AP activity was detected in the respective Francisella, Klebsiella, and Salmonella bacterial lysates.

**AP inhibition assay**

The effect of *F. novicida* lysate on exogenously added TNAP from bovine Kidney unless specified otherwise (all AP preparations procured from Sigma Chemical Co., St. Louis, MO) was determined using 4-MUP as substrate. Briefly, TNAP assay reaction mixtures contained 90 µl 10 mM Tris buffer (pH 7.4), 7 µl TNAP (25 µg), and 3 µl crude lysate (100 µg protein). Reaction mixtures were shaken continuously at 37 °C for 4 h after which time respective assay tubes were transferred to ice slurry. To each reaction mixture, substrate solution (900 µl AP assay buffer containing 5 mM 4-MUP) was added followed by incubation at 37 °C for 15 min. Reactions were stopped by addition of 2 ml 0.1 M dibasic potassium phosphate, and fluorescence measured using a Quantech fluorometer (Thermo Scientific, Rockford, IL, filter settings $\lambda_{\text{excitation}} = 345$ nm, $\lambda_{\text{emission}} = 440$ nm). The assay was linear with respect to time and protein for at least 20 min. Control assay mixtures were carried out in identical fashion as described above except 3 µl 10 mM Tris buffer pH 7.4 was added in lieu of Francisella cell lysate supernatant material. Quantitation of hydrolysis of 4-MUP was achieved using a 4-MU standard curve. One unit inhibitory factor is defined as a 1 % reduction of TNAP activity following incubation with 100 µg bacterial lysate protein.

**Fractionation of bacterial lysate by native PAGE and AP inhibition assay**

Bacterial lysate (100 µg) was loaded onto 4-15 % Tris-glycine gradient gels, and run under native conditions for 2 h at 180 V. The gel was cut into...
2-mm segments from top to bottom, and each segment was resuspended in 100 µl 10 mM Tris buffer, pH 7.4, homogenized on ice using a micro-glass tissue homogenizer, and centrifuged at 30,000 x g to sediment the acrylamide. Eluted protein was transferred to a clean assay tube for determination of AP inhibition as previously described but modified as below. The AP inhibition assay was carried out in triplicate, each assay tube containing 25 µl gel eluate, 25 µg TNAP (7 µl) and brought to a final volume of 100 µl with 10 mM Tris buffer, pH 7.4. Eluate from gel segments from a gel run under identical conditions but with no bacterial lysate served as control.

**DEAE anion exchange chromatography**

Anion exchange chromatography was carried out at 5 °C. Francisella (Fn) lysate (500 µl, 7.8 mg total protein) was added to DEAE slurry equilibrated in 10 mM Tris buffer, pH 7.4, thoroughly mixed (end-over-end) for 4 h at 5 °C after which time the resin was transferred to a 0.2 x 7 cm glass column. The column was washed with 2 column volumes equilibrating buffer (approximately 2.5 ml breakthrough material), and bound protein batch eluted using 50-200 mM NaCl in equilibrating buffer. Breakthrough material (100 µl), and respective salt eluates (100 µl) were diluted to 1 ml with 10 mM Tris buffer, pH 7.4, and concentrated to 100 µl using a Centricon Centrifugal Filter device (3000 dalton molecular weight cut off, Millipore, Billerica, MA).

**Mass spectrometry analysis-DEAE**

Breakthrough material and respective NaCl batch eluates were analyzed by native-PAGE (4-15 % gradient gel) using the method of Laemmli (19). Coomassie blue-stained bands were excised, digested in situ with trypsin (Trypsin Profile IGD Kit; Sigma Chemical Co.), and resulting peptides analyzed by capillary HPLC-electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) using a Thermo Fisher LTQ mass spectrometer (Department of Biochemistry, The University of Texas Health Science Center at San Antonio). Mass spectra were searched against the Swiss-Prot database by means of Mascot (Matrix assessment of probabilities of peptide and protein assignments by Scaffold, Proteome Software, Portland, OR). Significance thresholds for peptide and protein assignments were 95 and 99 % respectively, with a minimum of two peptides required for protein identification. Summarized in Table 1 are mass spectrometric data derived in this study.

**Immunoprecipitation and western blot analysis**

Amine activated resin (Thermo scientific, Rockford, IL) was coupled with 10 µl anti-alkaline phosphatase antibody (R and D Systems, Inc., Minneapolis, MN). Francisella lysate (360 µl, 500 µg protein) and TNAP (40 µl, 142 µg) were mixed together with antibody coupled beads and left at 5 °C for 6-8 h. Beads were washed five times with IP/lysis (Thermo Scientific) wash buffer followed by elution with IP elution buffer (Thermo Scientific). Co-immunoprecipitated proteins were separated on 4-15 % SDS polyacrylamide gels (BioRad Laboratories) and visualized either by coomassie blue staining or western blotting using anti-AP (R&D System, Minneapolis, MN), anti-DnaK (kindly provided by Dr. Jorge Benach, State University New York, Stony Brook), anti-GroEL (kindly provided by Dr. Daniel Clemens, University of California at Los Angeles), and anti-HtpG (kindly provided by Dr. Carol Gross, University of California San Francisco) antibodies. Following transfer of proteins to a polyvinylidene difluoride (PVDF) membrane (Amersham Hybond-P; GE Healthcare, Little Chalfont, UK), membranes were probed with -AP, -dnaK, -groEL, and -Htpg antibodies. Blots were developed following incubation with appropriate dilutions of horseradish peroxidase-conjugated secondary antibody using ECL Western blotting reagents (GE Healthcare, Little Chalfont, UK). Chemiluminescence was measured by autoradiography using Kodak XAR film (Eastman Kodak, Rochester, NY).

**Effect of antibodies and ATP on alkaline phosphatase inhibitory activity.** Francisella lysate was incubated with anti-DnaK (2.5, 5, 12.5, and 25 µg), anti-GroEL (25 µg), and anti-HtpG (25 µg) antibodies for 2 h at room temperature with continuous shaking. Following incubation, AP inhibition was determined as described above. Effect of ATP
on *F. novicida*-mediated AP inhibition was determined in triplicate, each assay tube containing 3 µl crude lysate, 25 µg TNAP (7 µl), and brought to a final volume of 100 µl with/without 90 µM 4mM ATP-MgSO$_4$ (dissolved in 10 mM Tris buffer, pH 7.4). A 20 µl aliquot was removed from above reaction mixtures for zymogram and western blot analyses.

**Statistical Analysis**—The Student’s *t* test was used to determine statistical significance. All data are presented as mean values ± the respective standard deviation.

**RESULTS**

**In vivo and in vitro inhibition of alkaline phosphatase activity**—We have previously reported that *F. novicida* rapidly disseminated to liver following intranasal (i.n.) challenge (7). We further assessed acute damage to this organ by analyzing a panel of liver function proteins in the plasma following *Francisella* infection. As shown in Fig. 1A, while albumin amount remained unaffected, aspartate aminotransferase (AST) activity was elevated 4-fold by 72 h compared to uninfected mice (time = 0 hr). Alanine aminotransferase (ALT) activity was observed to increase gradually as the infection progressed consistent with increased bacterial burden in the liver (20). Interestingly, plasma AP activity was significantly reduced as early as 48 h post challenge and the enzymatic activity decreased from 200 international units per liter (IU/l) at 24 h to 50 IU/l by 72 h post challenge in contrast to most bacterial and viral infections which are associated with increased plasma AP activity.

Under identical conditions used in this study, enumeration of *F. novicida* bacteria in cell free plasma indicated the presence of few if any organisms at 24 h post challenge, but 1.0 x 10$^7$ and 3.5 x 10$^7$ cfu/ml blood at 48 and 72 h, respectively (7). These burdens are consistent with little to no drop in AP activity reported here at 24 h, and 50 and 75 % decreased plasma AP activity at 48, and 72 h post challenge, respectively (Fig. 1A). Furthermore, the bacterial burden in the lungs 24, 48, and 72 h post challenge were 6, 8, and ∼5 log cfu/gm of lung tissue, respectively (7). Dissemination of organisms from the lungs (initial site of exposure) to secondary tissues, i.e., liver, the primary source of AP synthesis, is apparent by the bacterial burden increasing from 10$^5$ cfu/gm tissue at 24 h post challenge to 10$^6$, and 10$^8$ cfu/gm tissue at 48, and 72 h, respectively. Ray et al. observed similar Schu S4 dissemination from the lungs to the liver by 72 h post challenge to that observed here using *F. novicida* (21). Consistent with the original observations of Hambleton and coworkers (22,23), plasma AP activity was observed significantly reduced, i.e., ∼40 and 70 % at 48 and 72 h, respectively, in mice challenged i.n. with human virulent *Francisella* (Fig 1B).

There are 3 major AP isozymes in mammals. In order to determine which isoenzyme was affected following *Francisella* infection, tissue nonspecific AP (TNAP) enriched from bovine liver and kidney as well as calf intestinal AP (CIP), and human placental AP (PLAP) were assayed following incubation with *Francisella* (*Fn*) lysate. As shown in Fig. 1C, *Fn* lysate inhibited less than 6 % CIP and PLAP activities but significantly inhibited TNAP (27 % liver and 38 % kidney isoforms), strongly suggesting that TNAP is the major AP isozyme inhibited by *Francisella* infection.

**Specificity of Inhibition of Plasma Alkaline Phosphatase**—In order to determine if the reduction of AP activity was specific to *Francisella* infection, we also examined plasma prepared from mice challenged intranasally with 400 cfu of a related pneumonic Gram-negative organism, *K. pneumoniae*. As shown in Fig. 2A, plasma AP activity was relatively unchanged up to 72 h after *K. pneumoniae* challenge. In contrast, there was a marked reduction (70 %) of AP in plasma from both *F. novicida* (400 cfu), and *F. tularensis* LVS (400 cfu) infected mice by 72 h post challenge (Fig. 2A). Alkaline phosphatase enzymatic assays correlated with zymogram analysis showing no significant change in band intensity associated with hydrolysis of 4-MUP to 4-MU during the course of the *K. pneumonia* infection. In contrast, a marked reduction of AP intensity in LVS, and *Francisella* (*Fn*) infected plasma was observed...
as the infection progressed (Fig. 2B) suggesting reduction of plasma AP activity may be specific to Francisella infection. Western blot analysis of plasma prepared from PBS mock treated mice after 72 h using anti-AP antibody revealed a single dark band at ~70 kDa which decreased in intensity in the plasma of LVS infected animals (Fig. 2C) indicating decreased plasma AP protein following LVS infection may account for the observed reduction of AP enzymatic activity.

Gel electrophoretic characterization of inhibitory factor in bacterial lysate-In an attempt to identify the protein specific to Francisella responsible for AP inhibition, lysates from F. novicida, a related pneumonic Gram negative organism, i.e., K. pneumoniae, and an unrelated Gram negative enteric, i.e., S. typhimurium were coomassie blue stained following PAGE under non-denaturing conditions as shown in Fig. 3A. Although some differences in protein profiles were observed comparing the respective lanes, an identical gel was loaded with 100 µg Fn lysate protein (37 inhibitory units), K. pneumonia, and S. typhimurium, and run under identical conditions. The gel was cut in 2 mm segments, and the respective segment eluates evaluated for inhibition of TNAP activity. As shown in Fig. 3B, TNAP inhibitory proteins electrophoresed as a broad, heterogeneous peak ranging in molecular weight from approximately 72 to 170 kDa (closed circles) with majority of inhibition at ~130 kDa. Although the gel was cut from top to bottom, the profile shown in Fig. 3B represents only the inhibitory species eluted from the respective gel segments. Summation of inhibitory units across the profile accounted for approximately 31.5 of the 37 inhibitory units applied resulting in ~85 % recovery. No inhibition of TNAP activity was observed for gel segment eluates from K. pneumoniae and S. typhimurium. Consistent with decreasing intensity of AP activity band observed in the zymogram and western blot analyses of plasma (Fig. 2B and 2C), western blot analysis of TNAP incubated with Francisella (Fn) lysate resulted in a band of considerable less intensity (TNAP + Fn) compared to the control (TNAP - Fn) with no observable degradation products (Fig. 3C). No band was observed in the absence of TNAP (Fig. 3C). Inclusion of a protease inhibitor cocktail (Roche diagnostics, Indianapolis, IN) was inconclusive since the cocktail inhibited TNAP in the absence of lysate (data not shown).

Fractionation of Francisella novicida inhibitory factor(s) by DEAE anion exchange chromatography-In order to further purify and characterize the inhibitory component(s), Francisella cell lysate (7.80 mg protein equivalent to 1,700 starting Units inhibitory factor) was loaded onto a DEAE anion exchange column. Of the protein applied to the column, 1.80 mg did not exchange with the resin coming through in the column breakthrough. Elution of bound protein using increasing concentrations of NaCl resulted in the removal of 3.76 mg total protein with a total recovery of protein inclusive of breakthrough protein of 5.54 mg (~70 %). Assay of breakthrough material and salt eluates for inhibition of TNAP activity revealed the inhibitory factor to elute from 50 through 200 mM NaCl with no inhibitory factor found in either the breakthrough or 25 mM NaCl eluate. Following removal of salt by Centricon filtration/concentration, the maximum number of inhibitory units (62.4) was observed to elute in the presence of 150 mM NaCl corresponding to ~39 % of the total amount recovered following elution but only ~7 % of the starting inhibitory units (Fig. 4A). Analysis of the respective eluates by PAGE under non-reducing conditions indicated enrichment of a band of approximate 130 kDa in the 150 mM eluate (Fig. 4B). The 130 kDa band was excised, subjected to mass spectrophotometric analysis, and identified as the molecular chaperone heat shock protein DnaK (Hsp70)(cf. Table 1). A dark staining band at approximately 150 kDa was observed in starting Francisella lysate material (Fn), breakthrough (BT), and salt elutes (100 and 150 mM) but was not analyzed since assay of breakthrough material exhibited no AP inhibitory activity.

Interaction of Francisella DnaK with TNAP – To confirm DnaK interaction with TNAP, a TNAP-Francisella (Fn) lysate complex was pulled down using anti-AP antibody coupled to AminoLink Coupling Resin (αAP:ACR).
Protein complex captured by αAP:ACR was analyzed by PAGE under denaturing conditions, and 3 distinct protein bands ranging from ~60 to ~90 kDa were visible after coomassie blue staining (Fig 5A, middle lane). Binding of these 3 proteins to TNAP was specific since none of these F. novicida proteins were captured by αAP:ACR when the resin was incubated with Francisella lysate in the absence of TNAP (Fig. 5A, right lane). To identify these 3 TNAP binding proteins, bands were excised from the gel, trysin digested, and subjected to mass spectroscopic proteomic analysis. As expected, DnaK (~90 kDa) was identified in the upper band along with the identification of GroEL (~70 kDa), and HtpG (~60 kDa) in the middle, and lower band, respectively (proteomic data summarized in Table 1). Immunoblotting with anti-TNAP, -DnaK, -GroEL, and -HtpG antibodies further confirmed the presence of the respective proteins in the TNAP binding complex (Fig. 5B). Proteomic analysis did not identify TNAP; however, western blot analysis did reveal the presence of TNAP in the αAP:ACR pull down (Fig. 5B).

To further characterize inhibition of TNAP by these 3 identified proteins, we used corresponding antibodies (anti-DnaK, -GroEL, and -HtpG) to compete for TNAP binding and/or neutralization of AP inhibition. Specifically, Fn lysate was pre-incubated with 0, 2.5, 5, 12.5, and 25 μg anti-DnaK antibody for 2 h followed by 4 h reaction with TNAP. Alkaline phosphatase activity was assayed and results indicated pre-incubation of anti-DnaK antibody with Fn lysate markedly reduced AP inhibition in a dose-dependent manner with up to 80 % reduction of TNAP inhibitory activity by 25 μg/ml of anti-DnaK antibody (Fig. 6A, 25 μg/ml α-DnaK). Abrogation of F. novicida mediated AP inhibition by anti-DnaK antibody is specific since Fn lysate pre-incubated with heat denatured anti-DnaK antibody (25hi in Fig. 6A) or IgG isotype (data not shown) has essentially no effect on AP inhibition. Also, anti-DnaK antibody alone did not alter TNAP enzymatic activity (data not shown). Incubation of TNAP with 25 μg anti-GroEL (α-GroEL) or anti-HtpG (α-HtpG) antibodies had no significant effect (Fig. 6A), and increasing the antibody concentration to 75 μg/ml resulted in little (3-5 %) reduction of TNAP inhibition (data not shown). Additionally, zymogram analysis (Fig. 6B) of anti-DnaK antibody incubation with TNAP and lysate was shown to be protective (25 μg/ml) in a dose-dependent manner (2.5 μg/ml being less protective) of TNAP in comparison to no anti-DnaK antibody (0 μg/ml). Collectively, F. novicida heat shock proteins (DnaK, GroEL, and HtpG) appear to form a complex that binds to TNAP; however, only DnaK plays a role in AP inhibition.

Effect of ATP on inhibition of TNAP by Francisella lysate-DnaK has a N terminal ATPase, and C terminal substrate binding domains (24,25). Given that binding of ATP alters the conformational state of DnaK resulting in a low-affinity state, and subsequent release of the substrate, e.g. TNAP (26), we assessed the effect of ATP on inhibition of TNAP by Fn lysate. As shown in Fig. 7A, TNAP incubated in the presence of added ATP and lysate (TNAP + Fn lysate + ATP) exhibited essentially the same activity as that observed for TNAP alone. In contrast, TNAP activity in the presence of Fn lysate but absence of ATP was reduced ~ 32 %. This is corroborated in the zymogram analysis. Furthermore, addition of ADP and AMP had no effect on TNAP inhibition by Fn lysate (data not shown). To further examine whether ATP reverses TNAP inhibition by Fn lysate is due to reduction of TNAP binding to DnaK by ATP, we used αAP:ACR to capture the TNAP complex formed from the Fn lysate and TNAP mixture in the presence and absence of ATP. As shown in the immuno blot analysis of the resin captured TNAP complex (Fig. 7B), an equivalent amount of TNAP was recovered from the reaction with or without ATP; however, less DnaK was detected when ATP was present. Collectively, these results further support DnaK as being the Fn lysate component that binds to and inhibits TNAP.

DISCUSSION

Francisella tularensis is a highly infectious bacterium, since inhalation of only a few organisms can cause severe disease and death. Despite the high mortality rate in untreated
individuals, little is understood regarding *F. tularensis* virulence factors, or the innate and adaptive immune responses operating at the sites of primary infection. Because *F. tularensis* colonizes and causes severe disease in the liver, an important aspect of virulence is related to the ability of the organism to survive and multiply inside of hepatic cells.

In this study, we demonstrate that the plasma of *F. tularensis* challenged animals exhibited marked elevation in both AST and ALT enzyme activity indicative of liver damage by 72 h as previously reported (20). The temporal increase of these enzymes coincided with a significant decrease of AP in the plasma of *Francisella* infected mice. This reduction of host AP expression was observed across all *Francisella* species and subspecies, but was not apparent with another Gram-negative pneumonic pathogen, *K. pneumoniae*. Our results are in agreement with those of Hambleton et. al. (22,23), who observed a significant reduction of circulating AP in rabbits and monkeys infected by aerosolization, or intraperitoneal challenge with *F. tularensis* type A (SCHU S4 strain).

*Francisella* induced reduction of AP activity was further characterized using an *in vitro* assay and commercially available AP preparations. *Francisella* lysate significantly inhibited TNAP, the major AP isozyme present in mouse plasma. Bacterial lysates prepared from *K. pneumoniae* and *S. typhimurium* indicated no such inhibition suggesting inhibition to be *Francisella* specific consistent with *in vivo* observations. Identification of the inhibitory factor was achieved using PAGE, DEAE anion exchange chromatography, proteomic, and immunologic means revealing the presence of a complex comprised of heat shock proteins DnaK, GroEL, and HtpG. Incubation of bacterial lysate with increasing amounts of anti-DnaK resulted in dose-dependent reduction of TNAP inhibition; whereas, anti-GroEL and -HtpG had no significant effect.

In order to assess the role of DnaK in inhibition of plasma AP activity, we initially utilized the DnaK mutant FTN_1284 of the transposon library of Gallagher, *et al.* (27). However, we confirmed by overlap extension PCR using primers specific for the DnaK gene, and western blotting with anti-DnaK antibody that although the transposon was present, it appears not to be in the DnaK gene. An attempt to generate a DnaK mutant by homologous recombination was not successful suggesting DnaK may be essential for *Francisella* growth at 37 °C.

Consistent with the presence of an N-terminal ATPase domain in Hsp70 and the proposed DnaK chaperone cycle, ATP appears to significantly reduce inhibition of TNAP by *Francisella* lysate. Binding of ATP at the ATPase domain of Dnak has shown to trigger the release of substrate by the C terminus substrate binding domain (28). This is consistent with less *Francisella* DnaK bound to TNAP in the presence of ATP. Alkaline phosphatase of plasma was observed to be significantly inhibited (approximately 75 %) 72 h *post* infection; whereas, TNAP inhibition using the *in vitro* assay was 40 % maximum. Because bacterial cell lysate is the source of AP inhibitory activity, the 40 % inhibitory maximum could arise from cell lysate endogenous ATP thus reducing binding of DnaK. Additionally, the high turnover number exhibited by mammalian AP gives a greatly exaggerated impression of the amount of phosphatase protein actually present in a given tissue (29). Thus, the amount of DnaK present could significantly exceed that of plasma AP resulting in greater inhibition than that observed for the *in vitro* assay (25 µg TNAP protein). Inorganic phosphate has been shown to inhibit TNAP (30). However, using the standard clinical assay for TNAP with high concentration of artificial substrates (PNPP and 4-MUP), and diluted plasma and bacterial lysate in both *in vivo* and *in vitro* TNAP assays respectively, attainment of V_max is achieved due to dilution of P_i well below threshold inhibitory levels (30). Incubation of ATP, ADP, or AMP alone with TNAP had no effect upon TNAP activity.

The nature of the interaction of TNAP with DnaK was assessed using western blot analysis. Decreased antibody binding as well as enzymatic activity could arise from
conformation alteration consistent with DnaK’s ‘remodeling’ function or simple blocking of the TNAP epitope and/or catalytic site preventing binding of substrate. Heat shock proteins do not appear to have proteolytic activity, but have been shown to be associated with degradation of proteins (31,32). Associated with DnaK is protease La. Although, a Francisella La mutant lysate (prepared using a Francisella mutant library kindly provided KK by Dr. Colin Manoil, University of Washington, Seattle, WA) had no effect on inhibition of TNAP (data not shown), the involvement of other proteases cannot be ruled out.

In a comparative proteomic profiling of culture filtrate proteins of *F. tularensis* subsp. *tularensis*, strain SCHU S4 and attenuated *F. tularensis* subsp. *holariica*, Konecna et al., identified the most abundant group of culture filtrate proteins, *i.e.*, secreted to include a group of heat-shock proteins (GroES, GroEL, and DnaK) which have previously been demonstrated to be of importance for the ability of *F. tularensis* to survive and/or multiply inside host cells, suggesting that stress responses are of significance for the virulence of *Francisella* (33,34). These chaperone proteins are in general cytoplasmic proteins and none of the proteomic prediction algorithms suggested that they should be found in the extracellular space. However, recent studies suggest that they may be membrane associated or secreted in other bacteria (35,36). Pierson and coworkers have reported DnaK to be present in outer membrane vesicles of *F. novicida* suggesting yet another possible role in virulence (37). Interestingly, such altered and unexpected localization of proteins is often a hallmark of ‘moonlighting’ proteins, proteins possessing multiple apparently unrelated functions performed by one polypeptide chain (38). Since the diverse functions of a protein are frequently associated with its cellular location, a protein’s function in the cytosol may differ from that if located on the cell envelope, in vesicles, and/or the secretome, and could be implicated in virulence, *i.e.*, altering of the extracellular environment. The mechanism by which DnaK mediates the inhibition of TNAP remains to be elucidated.

Release of DnaK into the extracellular milieu and inhibition of host plasma AP could promote an inflammatory response advantageous to *Francisella*. Recently, Fraley and coworkers demonstrated profound effects on cellular composition and morphology in *Pseudomonas aeruginosa* polyphosphate kinase mutants (39). The *Francisella* poly P kinase gene is induced intracellularly and is required for intracellular growth and virulence (40). Polyphosphate is an inorganic, linear polymer of orthophosphate units linked by phosphoanhydride bonds and has been extensively studied in prokaryotes and lower eukaryotes where it functions in basic metabolism, stress responses, and as a structural component (41). In like fashion to DnaK, large-scale release into the plasma compartment of poly P via bacterial cell lysis or secretion could function as a proinflammatory mediator by activating the plasma contact activation, or so-called “intrinsic” coagulations system (42). The pathology of the pulmonary tularemia sepsis syndrome is characterized by wide dissemination of necrotic foci with histolytic inflammation, and pyogranulomas, accompanied by fibrin deposition, hemorrhage, and vascular inflammation (43). These pathologic changes are consistent with coagulation system activation. Coagulation activation and fibrin deposition may be advantageous to *Francisella in vivo* as a means to isolate foci of infected tissue from immune surveillance, and thus allowing bacterial replication and survival. Inhibition of AP by DnaK would facilitate this process by decreasing poly P clearance. Persistence of *Francisella in vivo* has been shown not to correlate with the mere ability to induce a protective immune response (44). Thus, the release of bacterial proteins/metabolites via secretion and/or cell lysis, *i.e.*, cell death may constitute in finality an orchestrated advantage.
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FOOTNOTES

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FIGURE LEGENDS

Figure. 1: Inhibition of murine alkaline phosphatase activity by Francisella spp. (A) BALB/c mice (3 per group) were challenged intranasally (i.n.) with 400 cfu F. novicida. Mice were bled at the indicated time points and plasma albumin content, transaminase (ALT and AST), and AP activities were determined using an Olympus AU640e Chemistry Immuno Analyzer. Enzyme activity was reported as IU/l (International Units per liter using PNPP as substrate) and albumin content g/dl (grams per deciliter). Mean values ± SD are shown for all experiments. Significant differences in enzymatic activities between mice prior to (0 h) and post F. novicida challenge (48 and 72 h) are indicated (*p < 0.05, **p < 0.01). Results are representative of three independent experiments. (B) BALB/c mice (3-5 per group) were challenged i.n. with either 100 cfu Type A or B Francisella. Mice were bled at 24, 48, and 72 h post challenge. Plasma was prepared and assayed using PNPP substrate as previously described under ‘Experimental Procedures’. Mean values ± SD are shown for all experiments. Significant differences in plasma AP activities between mice prior and post bacterial challenge are indicated (*p < 0.05, **p < 0.01). (C) The inhibitory effect of Francisella lysate supernatant material on liver and kidney tissue nonspecific AP (TNAP), calf intestinal AP (CIP), and placental AP (PLAP) isoenzymes was determined in triplicate as previously described under ‘Experimental Procedures’ using 4-methylumbelliferyl phosphate (4-MUP) as substrate. Mean values ± SD are shown for all experiments. Significant differences in AP activities are indicated (*p < 0.05, **p < 0.01).

Figure. 2: Specificity of inhibition of plasma alkaline phosphatase. BALB/c mice (3-5 per group) were challenged intranasally with 400 cfu of either K. pneumonia, F. holarctica (LVS), or F. novicida. Mice were bled prior to challenge (0 h) and at 24, 48, and 72 h post challenge. (A) Plasma AP activity was measured spectrophotometrically with PNPP substrate and reported as pmoles/min/µl plasma. Mean values ± SD are shown for all experiments. Significant differences in plasma AP activities are indicated (*p < 0.05, **p < 0.01). (B) Respective plasma samples were subjected to polyacrylamide gel electrophoresis and AP activity visualized under UV using 4-MUP substrate as previously described under ‘Experimental Procedures’. (C) Representative western blot analysis of PBS mock treated and LVS infected plasma (72 h) using anti-AP antibody. β-actin detected by an anti-actin antibody was used as a protein loading control (M$\alpha$ = 42 kDa).

Figure. 3: Fractionation of Francisella novicida inhibitory factor(s) by polyacrylamide gel electrophoresis. (A) Bacterial lysate protein (100 μg) obtained from early stationary phase cultures of F. novicida (Fn), K. pneumonia (Kn), and S. typhimurium (St) along with molecular weight standards (Std) were separated on a 4-15 % gradient gel, and stained with coomassie blue. (B) A similarly prepared gel but without staining was cut in 2 mm segments (numbered from top to bottom). Protein was eluted from the respective gel segments, incubated with TNAP for 4 h, and hydrolysis of 4-MUP carried out as previously described under ‘Experimental Procedures’. Reduction of total TNAP activity per gel segment was calculated as follows: TNAP + respective eluate/ TNAP control x 100. (C) Reduction of TNAP protein following incubation (4 h) with total Francisella lysate (Fn) was visualized by western blot analysis using an anti-AP antibody as previously described under ‘Experimental Procedures’.

Figure. 4: Fractionation of Francisella novicida inhibitory factor(s) by DEAE anion exchange chromatography. (A) Total F. novicida lysate was loaded onto a DEAE anion exchange column and fractionated as previously described under ‘Experimental Procedures’. Bound protein was eluted using increasing concentration of NaCl (50, 100, 150, and 200 mM). (B) Electrophoretic analysis of fractionated (coomassie blue stained) Francisella lysate was carried out as previously described under ‘Experimental Procedures’. Starting, unfractionated Francisella lysate is represented by Fn. Unbound protein, i.e., column breakthrough is represented by BT. Bound protein was eluted using increasing concentration of NaCl (50, 100, 150, and 200 mM), respectively. Inhibition of AP by each NaCl eluate
was measured following 4 h incubation with TNAP as previously described under ‘Experimental Procedures’.

**Figure. 5:** *Francisella novicida* DnaK, GroEL, and HtpG proteins were co-immunoprecipitated with TNAP using anti-TNAP antibody coupled beads. *F. novicida* (*Fn*) lysate (360 µl, 500 µg protein) mixed with TNAP (40 µl, 142 µg) or *Fn* lysate alone were incubated with anti-TNAP antibody coupled with AminoLink Coupling Resin for 6-8 h at 5 °C. Co-immunoprecipitated proteins were separated on 4-15 % SDS polyacrylamide gels and visualized either by (A) coomassie blue staining or (B) western blotting using anti-AP, anti-DnaK, anti-GroEL or anti-HtpG antibodies as described under ‘Experimental Procedures’.

**Figure. 6:** *Francisella novicida* mediated AP inhibition was abrogated by anti-DnaK antibody. (A) *F. novicida* cell lysate was incubated with TNAP in the absence (0) or presence of increasing concentration (2.5 to 25 µg/ml as indicated) anti-DnaK, anti-GroEL (25 µg/ml), anti-HtpG (25 µg/ml) or 25 µg/ml heat-inactivated anti-DnaK (25hi) antibodies for 4 h. Inhibition of AP was determined for each antibody treatment as previously described under ‘Experimental Procedures’. (B) Zymogram analysis of AP activity in the absence (0) or presence of anti-DnaK antibody (2.5 and 25 µg/ml).

**Figure. 7:** Exogenous ATP reduced *Francisella novicida* mediated AP inhibition. (A) *F. novicida* (*Fn*) cell lysate was incubated with TNAP in the absence or presence of 4 mM ATP-MgSO₄ for 4 h. Incubation of TNAP alone for 4 h was used as control. Alkaline Phosphatase (AP) activity was measured using 4-MUP substrate as previously described under ‘Experimental Procedures’ and reported as nmoles/min/mg. Shown below the inhibition profile is the zymogram analysis of TNAP +/- ATP. (B) Anti-TNAP antibody coupled with AminoLink Coupling Resin was mixed with *F. novicida* cell lysate material (500 µg protein) and TNAP (142 µg) in the presence and/or absence of 4 mM ATP for 6-8 h at 5 °C. Proteins captured by anti-TNAP antibody coupled resin were separated on a 4-15 % SDS polyacrylamide gel and DnaK co-immunoprecipitation with TNAP was analyzed by western blotting using anti-DnaK and anti-AP antibodies as described under ‘Experimental Procedures’.
Table 1: Summary of mass spectrophotometric data derived in this study. Protein bands of interest (Figs. 4B and 5A) were digested with trypsin and resulting peptides analyzed by capillary HPLC-electrospray ionization tandem mass spectrometry as described under ‘Experimental Procedures’.

| S. No | Identified Proteins                              | Locus Tag | NCBI Identifier | % of sequence coverage | Molecular Weight (kDa) | Probability |
|-------|--------------------------------------------------|-----------|-----------------|-------------------------|------------------------|-------------|
| 1     | Heat shock protein DnaK                          | FTN_1284  | gi|118497869 | 46.0                  | 69                      | 95%         |
| 2     | Elongation factor Tu                             | FTN_1576  | gi|118498143 | 35.0                  | 43                      | 95%         |
| 3     | Fructose-1,6-bisphosphate aldolase               | FTN_1329  | gi|118497908 | 20.0                  | 38                      | 95%         |
| 4     | Valyl-tRNA synthetase                            | FTN_0214  | gi|118496828 | 11.0                  | 105                     | 95%         |
| 5     | Bifunctional purine biosynthesis protein PurH    | FTN_0177  | gi|118496791 | 13.6                  | 56                      | 95%         |
| 6     | Serine hydroxymethyltransferase                  | FTN_1259  | gi|118497844 | 5.0                   | 45                      | 95%         |
| 7     | Fumarate hydratase                               | FTN_0337  | gi|118496947 | 5.9                   | 55                      | 95%         |
| 8     | Phosphoribosylformyl glycinamidine synthase      | FTN_1699  | gi|118498260 | 2.8                   | 141                     | 95%         |
| 9     | AhpC/TSA family peroxiredoxin                    | FTN_0973  | gi|118497565 | 25.1                  | 22                      | 95%         |
| 10    | Glycine cleavage system aminomethyltransferase T | FTN_0505  | gi|118497104 | 8.9                   | 40                      | 95%         |
| 11    | UDP-glucose/GDP-mannose dehydrogenase            | FTN_1426  | gi|118497998 | 8.9                   | 49                      | 95%         |
| 12    | Cysteinyl-tRNA synthetase                        | FTN_0310  | gi|118496920 | 7.8                   | 52                      | 95%         |
| 13    | Aconitate hydratase                              | FTN_1623  | gi|118498186 | 6.0                   | 103                     | 95%         |
| 14    | Intracellular growth locus B protein             | FTN_1323  | gi|118497903 | 6.0                   | 59                      | 95%         |
| 15    | Glyceraldehyde-3-phosphate dehydrogenase         | FTN_1332  | gi|118497911 | 9.9                   | 38                      | 95%         |
| 16    | Protein chain elongation factor EF-Ts            | FTN_0228  | gi|118496841 | 9.6                   | 31                      | 95%         |
| 17    | Elongation Factor G                              | FTN_0237  | gi|118496850 | 11.8                  | 78                      | 95%         |
| 18    | Elongation factor P                              | FTN_0069  | gi|118496684 | 17.5                  | 21                      | 95%         |
Fig. 1

A

B

C

Running title: Alkaline phosphatase inhibition and Francisella survival
Fig. 2
Fig. 3
Fig. 4
Fig. 5
Fig. 6
Fig. 7
Francisella DnaK Inhibits Tissue Nonspecific Alkaline Phosphatase
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