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Francisella DnaK Inhibits Tissue-nonspecific Alkaline Phosphatase*

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Bernard P. Arulanandam[‡1], Senthilnath Lakshmana Chetty[‡1], Jieh-Juen Yu[‡], Sean Leonard[‡], Karl Klose[‡], Janakiram Seshu[‡], Andrew Cap[§], James J. Valdes[¶], and James P. Chambers[‡2]

From the [‡]Department of Biology, University of Texas, San Antonio, Texas 78249, the [§]United States Army Institute of Surgical Research, Fort Sam Houston, Texas 78234, the [¶]United States Army Edgewood Chemical Biological Command, Aberdeen Proving Ground, Maryland 21010

Background: Pulmonary *Francisella* infection resulted in reduction of plasma alkaline phosphatase activity.
Results: *Francisella* heat shock protein DnaK binds to alkaline phosphatase thus 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.

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 pneumoniae*) 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) was 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 the following: (i) *F. tularensis* subsp. *tularensis* (type A); (ii) *F. tularensis* subsp. *holarctica* (type B); (iii) *F. tularensis* subsp. *mediasiatica*; and (iv) *Francisella novicida* (1). Although type A and B strains are the most relevant in terms of human disease, *F. novicida* and the live vaccine strain (LVS)[3] *F. tularensis* (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 because most reported pathogen infections give rise to increased AP activity.

Alkaline phosphatase (orthophosphoric monoester phosphohydrolase, 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 as follows: 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 the 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.

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[1] Both authors contributed equally to this work.

[2] To whom correspondence should be addressed: Dept. of Biology, University of Texas, One UTSA Circle, San Antonio, TX. Tel.: 210-458-5663; Fax: 210-458-5658; E-mail: james.chambers@UTSA.edu.

[3] The abbreviations used are: LVS, live vaccine strain; AP, alkaline phosphatase; TNAP, tissue-nonspecific AP; 4-MU, 4-methylumbelliferone; 4-MUP, 4-methylumbelliferyl phosphate; i.n., intranasal.

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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—*F. 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* ($KPPR_1$ strain) (16), and *Salmonella typhimurium* (ATCC, strain 14028) were inoculated in trypticase soy broth supplemented with 0.1% (w/v) L-cysteine hydrochloride, 0.025% (w/v) sodium pyruvate, 0.025% (w/v) sodium metabisulfite, and 0.025% (w/v) ferrous sulfate. 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 NCI-Frederick, National Institutes of Health. 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 (LD_{50} <10 cfu) or *F. tularensis* type B (LD_{50} = 10 cfu) in 25 μl of phosphate-buffered saline (PBS) or with 400 cfu of *F. novicida* (LD_{50} = 10 cfu), LVS (LD_{50} = 2800 cfu), or *K. pneumoniae* (LD_{50} < 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). Respective plasma samples were centrifuged for 5 min at 5000 rpm, and aliquots were frozen at −20 °C until used.

Plasma Biochemical Assays—Plasma albumin content as well as alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase (AP) levels were measured 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 (μmole/min/liter or pmol/min/μl) in 96-well microplates by measuring the rate of hydrolysis of *para*-nitrophenyl phosphate (PNPP) (Sigma) or 4-methylumbelliferyl phosphate (4-MUP) (Sigma) as described previously (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 was 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 *para*-nitrophenol (0–60 nmol) or 4-methylumbelliferone (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 (Bio-Rad) and run under native conditions at 180 V for 2 h after which time the gel was washed three 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 of 0.10 M NH_4OH, 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 were harvested by centrifugation. Following suspension in 5 ml of 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 × *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), was determined using 4-MUP as substrate. Briefly, TNAP assay reaction mixtures contained 90 μl of 10 mM Tris buffer, pH 7.4, 7 μl of TNAP (25 μg), and 3 μl of crude lysate (100 μg of protein). Reaction mixtures were shaken continuously at 37 °C for 4 h after which time respective assay tubes were transferred to an ice slurry. To each reaction mixture, substrate solution (900 μl of 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 of 0.1 M dibasic potassium phosphate, and fluorescence was measured using a Quantech fluorometer (Thermo Scientific, Rockford, IL, filter settings $\lambda_{excitation}$ = 345 nm and $\lambda_{emission}$ = 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 of 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 of bacterial lysate protein.

Fractionation of Bacterial Lysate by Native PAGE and AP Inhibition Assay—Bacterial lysate protein (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 of 10 mM Tris buffer, pH 7.4, homogenized on ice using a micro-glass tissue homogenizer, and centrifuged at 30,000 × *g* to sediment the acrylamide. Eluted protein was transferred to a clean assay tube for determination of AP inhibition as described previously but modified as below. The AP inhibition assay was carried out in triplicate, each assay tube containing 25 μl of gel eluate, 25 μg of 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. *F. novicida* lysate (500 μl, 7.8 mg of total protein) was added to a 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 × 7 cm glass column. The column was washed with 2 column volumes of equilibrating buffer (~2.5 ml of breakthrough material), and bound protein was 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 mass cutoff, 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 and digested *in situ* with trypsin (Trypsin Profile IGD kit; Sigma), and the resulting peptides were analyzed by capillary HPLC-electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) using a Thermo Fisher LTQ mass spectrometer (Department of Biochemistry, University of Texas Health Science Center, 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, Inc., 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 the mass spectrometric data derived in this study.

Immunoprecipitation and Western Blot Analysis—Amine-activated resin (Thermo Scientific, Rockford, IL) was coupled with 10 μl of anti-alkaline phosphatase antibody (R&D Systems, Inc., Minneapolis, MN). *Francisella* lysate (360 μl, 500 μg of 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 (Bio-Rad) under denaturing conditions, 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, UCLA), 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 (Hybond-P, GE Healthcare), membranes were probed with anti-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). Chemiluminescence was measured by autoradiography using Kodak XAR film (Eastman Kodak Co.).

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 with each assay tube containing 3 μl of crude lysate, 25 μg of TNAP (7 μl), and brought to a final volume of 100 μl with/without 90 μl of 4 mM ATP/$MgSO_4$ (dissolved in 10 mM Tris buffer, pH 7.4). A 20-μl aliquot was removed from the 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 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. 1*A*, although the albumin amount remained unaffected, aspartate aminotransferase activity was elevated 4-fold by 72 h compared with uninfected mice (time = 0 h). Alanine aminotransferase activity was observed to increase gradually as the infection progressed which is 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/liter (IU/liter) at 24 h to 50 IU/liter by 72 h post-challenge, in contrast to most bacterial and viral infections that 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 1.0×10^3 and 3.5×10^3 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. 1*A*). Furthermore, the bacterial burden in the lungs 24, 48, and 72 h post-challenge was 6, 8, and ~5 log cfu/g 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 10^2 cfu/g tissue at 24 h post-challenge to 10^6 and 10^8 cfu/g tissue at 48, and 72 h, respectively. Ray *et al.* (21) observed similar Schu S4 dissemination from the lungs to the liver by 72 h post-challenge to that observed here using *F. novicida*. Consistent with the original observations of Hambleton *et al.* (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. 1*B*).

There are three major AP isozymes in mammals. To determine which isozyme was affected following *Francisella* infection, TNAP enriched from bovine liver and kidney as well as calf intestinal AP and human placental AP were assayed following incubation with *F. novicida* lysate. As shown in Fig. 1*C*, *F. novi-*

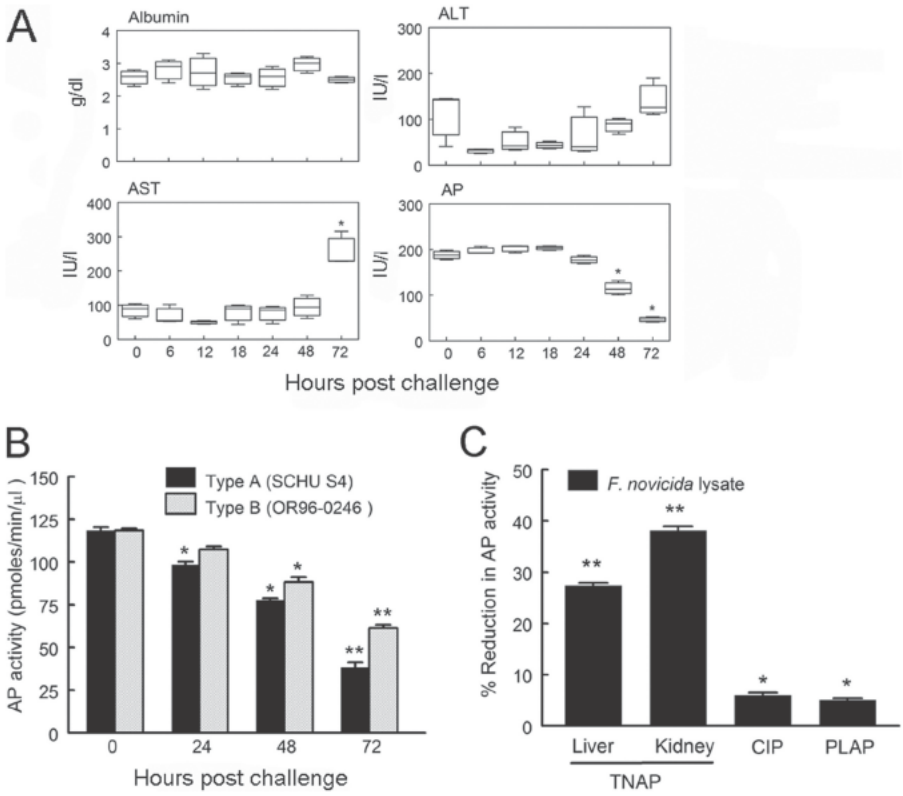

FIGURE 1. **Inhibition of murine alkaline phosphatase activity by *Francisella* spp.** *A,* BALB/c mice (three per group) were challenged i.n. with 400 cfu of *F. novicida*. Mice were bled at the indicated time points, and plasma albumin content, transaminase (*ALT* and *AST*), and AP activities were determined using an Olympus AU640e Chemistry Immuno Analyzer. Enzyme activity was reported as IU/liter (using PNPP as substrate) and albumin content g/dl. Mean values ± S.D. 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 (*, $p < 0.05$, Student's *t* test). Results are representative of three independent experiments. *B,* BALB/c mice (3–5 per group) were challenged i.n. with either 100 cfu of 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 described previously under "Experimental Procedures." Mean values ± S.D. are shown for all experiments. Significant differences in plasma AP activities between mice prior to and post-bacterial challenge are indicated (*, $p < 0.05$; **, $p < 0.01$). *C,* inhibitory effect of *Francisella* lysate supernatant material on liver and kidney TNAP, calf intestinal AP (*CIP*), and placental AP (*PLAP*) isozymes (equivalent units) was determined in triplicate as described previously under "Experimental Procedures" using 4-MUP as substrate. Mean values ± S.D. are shown for all experiments. Significant differences in AP activities are indicated (*, $p < 0.05$; **, $p < 0.01$).

cida lysate inhibited less than 6% calf intestinal AP and placental AP 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—To determine whether 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. 2*A*, 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. 2*A*). Alkaline phosphatase enzymatic assays correlated with zymogram analysis showed 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 *F. novicida* infected plasma was observed as the infection progressed (Fig. 2*B*) suggesting that 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 that decreased in intensity in the plasma of LVS infected animals (Fig. 2*C*), indicating that 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* and 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 nondenaturing conditions as shown in Fig. 3*A*. Although some differences in protein profiles were observed comparing the respective lanes, an identical gel was loaded with 100 µg of *F. novicida* 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 were evaluated for inhibition of TNAP activity. As shown in Fig. 3*B*, TNAP inhibitory proteins electrophoresed as a broad, heterogeneous peak ranging in molecular mass from ~72 to 170 kDa (*closed circles*) with the majority of inhibition at ~130 kDa. Although the gel was cut from top to bottom, the profile shown in Fig. 3*B* represents only the inhibitory species eluted from the respective gel segments. Summation of inhibi-

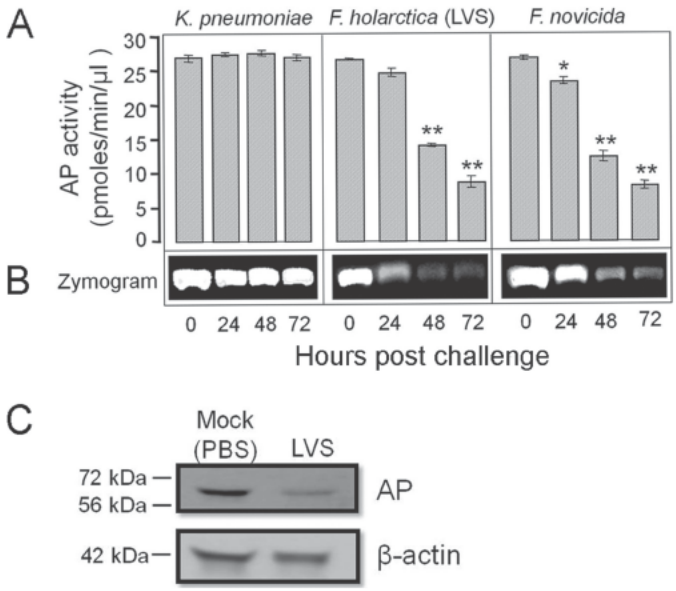

FIGURE 2. **Specificity of inhibition of plasma alkaline phosphatase.** BALB/c mice (3–5 per group) were challenged i.n. with 400 cfu of either *K. pneumoniae*, *F. holartica* (LVS), or *F. novicida*. Mice were bled prior to challenge (0 h) and at 24, 48, and 72 h post-challenge. *A*, plasma AP activity was measured spectrophotometrically with PNPP substrate and reported as pmol/min/μl plasma. Mean values ± S.D. 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 PAGE, and AP activity was visualized under UV light using 4-MUP substrate as described previously 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 (42 kDa).

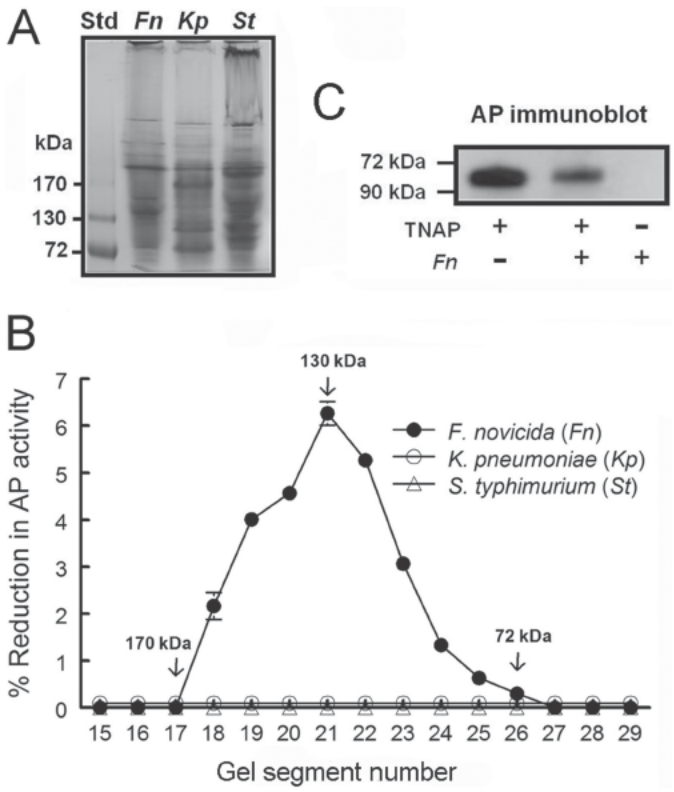

FIGURE 3. **Fractionation of *F. novicida* inhibitory factor(s) by PAGE.** *A*, bacterial lysate protein (100 μg) obtained from early stationary phase cultures of *F. novicida* (*Fn*), *K. pneumonia* (*Kp*), and *S. typhimurium* (*St*) along with molecular weight standards (*Std*) were separated on a 4–15% gradient gel and stained with Coomassie Blue. *B*, 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 and incubated with TNAP for 4 h, and hydrolysis of 4-MUP was carried out as described previously under "Experimental Procedures." Reduction of total TNAP activity per gel segment was calculated as follows: TNAP + respective eluate/TNAP control × 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 described previously under "Experimental Procedures."

tory units across the profile accounted for ~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. 2, *B* and *C*), Western blot analysis of TNAP incubated with *F. novicida* lysate resulted in a band of considerable less intensity (TNAP + *F. novicida*) compared with the control (TNAP − *F. novicida*) with no observable degradation products (Fig. 3*C*). No band was observed in the absence of TNAP (Fig. 3*C*). Inclusion of a protease inhibitor mixture (Roche Diagnostics) was inconclusive because the mixture inhibited TNAP in the absence of lysate (data not shown).

Fractionation of F. novicida Inhibitory Factor(s) by DEAE-Anion Exchange Chromatography—To further purify and characterize the inhibitory component(s), *Francisella* cell lysate (7.80 mg of protein equivalent to 1700 starting units of 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 of 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 to 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. 4*A*). Analysis of the respective eluates by PAGE under native conditions indicated enrichment of a band of ~130 kDa in the 150 mM eluate (Fig. 4*B*). 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 ~150 kDa was observed in starting *Francisella* lysate material, breakthrough, and salt eluates (100 and 150 mM) but was not analyzed because assay of breakthrough material exhibited no AP inhibitory activity.

Interaction of Francisella DnaK with TNAP—To confirm DnaK interaction with TNAP, a TNAP-*F. novicida* 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 three distinct protein bands ranging from ~60 to ~90 kDa were visible after Coomassie Blue staining (Fig. 5*A*, *middle lane*). Binding of these three proteins to TNAP was spe-

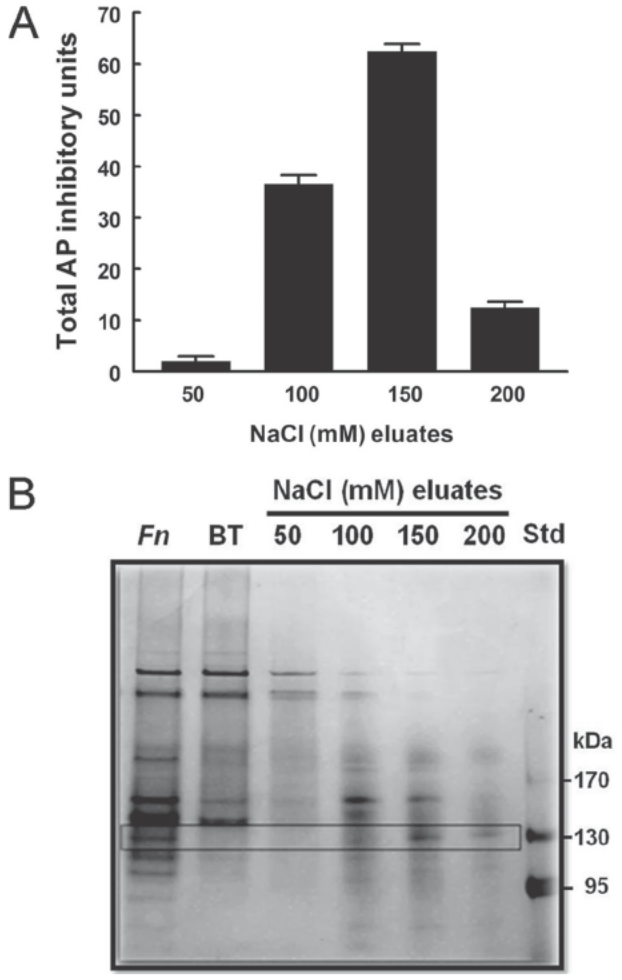

FIGURE 4. **Fractionation of *F. novicida* inhibitory factor(s) by DEAE-anion exchange chromatography.** *A,* total *F. novicida* lysate was loaded onto a DEAE-anion exchange column and fractionated as described previously under "Experimental Procedures." Bound protein was eluted using increasing concentrations of NaCl (50, 100, 150, and 200 mM). Inhibition of AP by each NaCl eluate was measured following a 4-h incubation with TNAP as described previously under "Experimental Procedures." *B,* electrophoretic analysis of fractionated (Coomassie Blue-stained) *Francisella* lysate was carried out as described previously under "Experimental Procedures." Unfractionated *Francisella* lysate is represented by *Fn.* Unbound protein, *i.e.* column breakthrough, is represented by *BT.*

cific because 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. 5*A*, *right lane*). To identify these three TNAP-binding proteins, bands were excised from the gel, trypsin-digested, and subjected to mass spectroscopic proteomic analysis. DnaK was identified in the upper band along with the identification of HtpG (~70 kDa) and GroEL (~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. 5*B*). Proteomic analysis did not identify TNAP; however, Western blot analysis did reveal the presence of TNAP in the αAP/ACR pulldown (Fig. 5*B*).

To further characterize inhibition of TNAP by these three identified proteins, we used the corresponding antibodies (anti-DnaK, -GroEL, and -HtpG) to compete for TNAP binding and/or neutralization of AP inhibition. Specifically, *F. novicida* lysate was preincubated with 0, 2.5, 5, 12.5, and 25 μg of anti-DnaK antibody for 2 h followed by a 4-h reaction with TNAP. Alkaline phosphatase activity was assayed, and results indicated preincubation of anti-DnaK antibody with *F. novicida* 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. 6*A*, 25 μg/ml α-DnaK). Abrogation of *F. novicida*-mediated AP inhibition by anti-DnaK antibody is specific because *F. novicida* lysate preincubated with heat-denatured anti-DnaK antibody (*25hi* in Fig. 6*A*) 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 of anti-GroEL (α-GroEL) or anti-HtpG (α-HtpG) antibodies had no significant effect (Fig. 6*A*), 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. 6*B*) 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 with 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 *F. novicida* lysate. As shown in Fig. 7*A*, TNAP incubated in the presence of added ATP and lysate (TNAP + *F. novicida* lysate + ATP) exhibited essentially the same activity as that observed for TNAP alone. In contrast, TNAP activity in the presence of *F. novicida* 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 *F. novicida* lysate (data not shown). To further examine whether ATP reverses TNAP inhibition by *F. novicida* lysate is due to reduction of TNAP binding to DnaK by ATP, we used αAP/ACR to capture the TNAP complex formed from the *F. novicida* lysate and TNAP mixture in the presence and absence of ATP. As shown in the immunoblots of the resin-captured TNAP complex (Fig. 7*B*), 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 *F. novicida* lysate component that binds to and inhibits TNAP.

DISCUSSION

F. tularensis is a highly infectious bacterium because 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

TABLE 1

Summary of mass spectrophotometric data derived in this study

Protein bands of interest (Figs. 4*B* and 5*A*) were digested with trypsin, and the resulting peptides analyzed by capillary HPLC-electrospray ionization tandem mass spectrometry as described under "Experimental Procedures."

Sample no.	Identified proteins	Locus tag	NCBI identifier	Sequence coverage	Molecular mass	Probability
				%	*kDa*	%
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		103	95
14	Intracellular growth locus B protein	FTN_1323	gi|118497903	6.0	59	95
15	Glyceraldehyde-3-phosphate dehydrogenase (phosphorylating)	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_069	gi|118496684	17.5	21	95
1	Heat shock protein DnaK	FTN_1284	gi|118497869	46.0	69	95
2	Heat shock protein GroEL	FTN_1538	gi|118498107	6.0	57	95
3	Heat shock protein HtpG	FTN_0266	gi|118496879	13.1	72	95

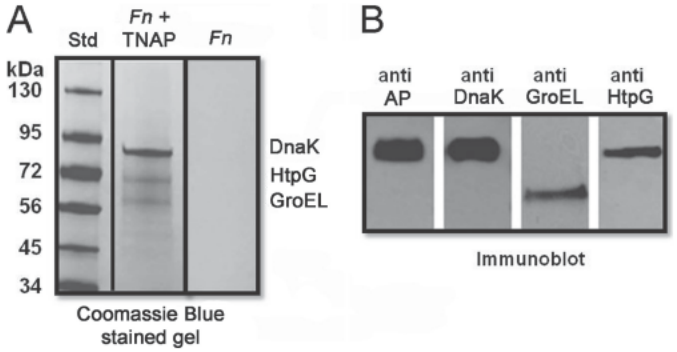

FIGURE 5. ***F. novicida* DnaK, GroEL, and HtpG proteins were co-immunoprecipitated with TNAP using anti-TNAP antibody-coupled beads.** *F. novicida* (*Fn*) lysate (360 μl, 500 μg of protein) mixed with TNAP (40 μl, 142 μg) or *F. novicida* lysate alone were incubated with anti-TNAP antibody coupled 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 Coomassie Blue staining (*A*) or Western blotting (*B*) using anti-AP, anti-DnaK, anti-GroEL, or anti-HtpG antibodies as described under "Experimental Procedures."

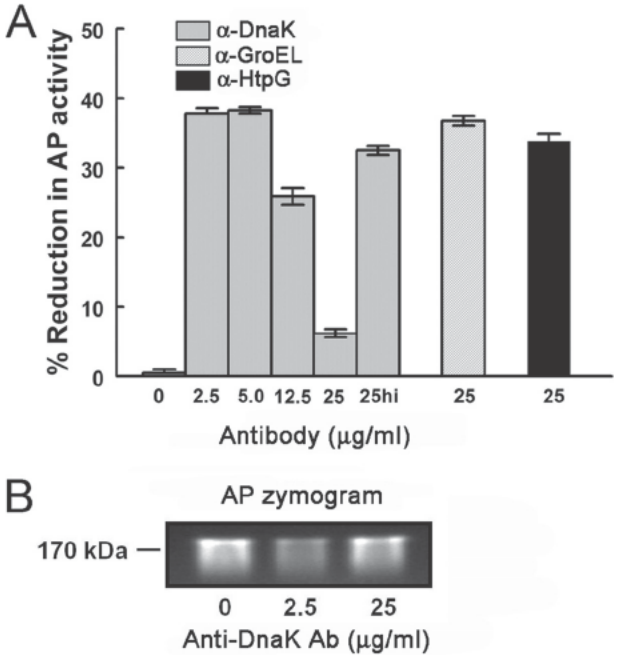

FIGURE 6. ***F. novicida*-mediated AP inhibition was abrogated by anti-DnaK antibody.** *A, F. novicida* cell lysate was incubated with TNAP in the absence (0) or presence of increasing concentrations (2.5 to 25 μg/ml as indicated) of anti-DnaK, anti-GroEL (25 μg/ml), anti-HtpG (25 μg/ml), or 25 μg/ml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

severe disease in the liver, an important aspect of virulence is related to the ability of the organism to survive and multiply inside hepatic cells.

In this study, we demonstrate that the plasma of *F. tularensis*-challenged animals exhibited marked elevation in both aspartate aminotransferase and aspartate aminotransferase enzyme activity indicative of liver damage by 72 h as reported previously (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 it 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 was *Francisella*-specific consistent with *in vivo* observations. Identification of the inhibitory factor was achieved using PAGE, DEAE-

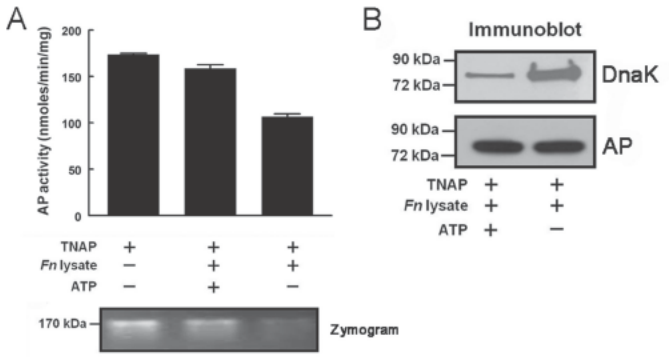

FIGURE 7. **Exogenous ATP-reduced *F. novicida*-mediated AP inhibition.** *A, F. novicida (Fn)* cell lysate was incubated with TNAP in the absence or presence of 4 mM ATP-$MgSO_4$ for 4 h. Incubation of TNAP alone for 4 h was used as control. AP activity was measured using 4-MUP substrate as described previously under "Experimental Procedures" and reported as nmol/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 of 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."

anion exchange chromatography, and 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.

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 been shown to trigger the release of substrate by the C-terminal 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 (~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 of 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 on 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 a conformation alteration consistent with the DnaK "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 to K. Klose 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. *holarctica,* Konecna *et al.* (34) identified the most abundant group of culture filtrate proteins, *i.e.* secreted to include a group of heat shock proteins (GroES, GroEL, and DnaK) that were previously 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). 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 *et al.* (37) have reported DnaK to be present in outer membrane vesicles of *F. novicida* suggesting yet another possible role in virulence. Interestingly, such altered and unexpected localization of proteins is often a hallmark of "moonlighting" proteins, proteins possessing multiple and apparently unrelated functions performed by one polypeptide chain (38). Because the diverse functions of a protein are frequently associated with it its cellular location, a function of the protein in the cytosol may differ from that 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 *et al.* (39) demonstrated profound effects on cellular composition and morphology in *Pseudomonas aeruginosa* polyphosphate kinase mutants. The *Francisella* polyphosphate 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 polyphosphate via bacterial cell lysis or secretion could func-

tion 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, thus allowing bacterial replication and survival. Inhibition of AP by DnaK would facilitate this process by decreasing polyphosphate 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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