**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 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.

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 703-0030-016), *Klebsiella pneumoniae* (KPPR1 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 metabisulfit, 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 (*L500 < 10 cfu*) or *F. tularensis* type B (*L500 = 10 cfu*) in 25 μL of phosphate-buffered saline (PBS) or with 400 cfu of *F. novicida* (*L500 = 10 cfu*), LVS (*L500 = 2800 cfu*), or *K. pneumoniae* (*L500 < 100 cfu*). Mice were bled at 0, 24, 48, and 72 h postchallenge, 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 MgCl2 and 1 mm ZnCl2). 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 for 5 min 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-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 (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 NH4OH, 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 45 s, 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 λexcitation = 345 nm and λemission = 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
Effect of Antibodies and ATP on Alkaline Phosphatase Inhibitory Activity—Francisella lysole 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 TNA (7 μl), and brought to a final volume of 100 μl with/without 90 μl of 4 mM ATP/MgSO₄ (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. 1A, 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 × 10³ and 3.5 × 10⁵ 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 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 10² cfu/g tissue at 24 h post-challenge to 10⁶ and 10⁸ 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. 1B).

There are three major AP isozymes in mammals. To determine which isozyme was affected following Francisella infection, TNA 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. 1C, F. novi-
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**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 AU640i 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 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 i.p. 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 showed no significant change in band intensity associated with hydrolysis of 4-MUP to 4-MU during the course of the *K. pneumoniae* infection. In contrast, a marked reduction of AP intensity in LVS and *F. novicida* infected plasma was observed as the infection progressed (Fig. 2B) 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. 2C), 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, *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. 3A. 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. pneumoniae*, 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. 3B, 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. 3B represents only the inhibitory species eluted from the respective gel segments. Summation of inhibi-
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![Graphs and images](http://www.jbc.org/)

**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. holarctica* (LV5), 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 nmol/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. pneumoniae* (Kp), and *S. typhimurium* (St) along with molecular weight standards (Std) were separated on a 4–15% gradient gel and stained with Coomassie Blue. β, similarly prepared gel without staining was cut in 2-mm segments (numbered from top to bottom). Protein was eluted from the respective gel segments and incubated with TNP for 4 h, and hydrolysis of 4-MUP was carried out as described previously under “Experimental Procedures.” Reduction of total TNP activity per gel segment was calculated as follows: TNP + respective eluate/TNP control × 100. C, reduction of TNP 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.”

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**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 TNP 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. Follow-

ing 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 native conditions indicated enrichment of a band of ~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 ~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 TNP.** To confirm DnaK interaction with TNP, a TNP-F. novicida lysate complex was pulled down using anti-AP antibody coupled to AminoLink Coupling Resin (aAP/ACR). Protein complex captured by aAP/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. 5A, middle lane). Binding of these three proteins to TNP was spe-
specific because none of these F. novicida proteins were captured by αAP/ACR when the resin was incubated with Francisella lyse in the absence of TNAP (Fig. 5A, right lane). To identify these three TNAP-binding proteins, bands were excised from the gel, trypsin-digested, and subjected to mass spectrometric 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. 5B). Proteomic analysis did not identify TNAP; however, Western blot analysis did reveal the presence of TNAP in the αAP/ACR pulldown (Fig. 5B).

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 lyse 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 lyse 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 because F. novicida lyse preincubated with heat-denatured anti-DnaK antibody (25h) 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. 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 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 lyse. As shown in Fig. 7A, 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 lyse 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. 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 F. novicida lyse 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
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-
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FIGURE 7. Exogenous ATP-reduced F. novicida-mediated AP inhibition. A. F. novicida (Fn) cell lysate was incubated with TNP in the absence or presence of 4 mM ATP-MgSO4 for 4 h. Incubation of TNP 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 TNP ± ATP. B, anti-TNP antibody coupled with AminoLink coupling resin was mixed with F. novicida cell lysate material (500 μg of protein) and TNP (142 μg) in the presence and/or absence of 4 mM ATP for 6–8 h at 5 °C. Proteins captured by anti-TNP antibody-coupled resin were separated on a 4–15% SDS-polyacrylamide gel, and DnaK co-immunoprecipitation with TNP 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 TNP 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 TNP 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 TNP in the presence of ATP. Alkaline phosphatase of plasma was observed to be significantly inhibited (~75%) 72 h post-infection; whereas, TNP 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 TNP protein). Inorganic phosphate has been shown to inhibit TNP (30). However, using the standard clinical assay for TNP with high concentration of artificial substrates (PNPP and 4-MUP) and diluted plasma and bacterial lysate in both in vivo and in vitro TNP assays, respectively, attainment of Vmax is achieved due to dilution of P, well below threshold inhibitory levels (30). Incubation of ATP, ADP, or AMP alone with TNP had no effect on TNP activity.

The nature of the interaction of TNP with DnaK was assessed using Western blot analysis. Decreased antibody binding as well as enzymatic activity could arise from a conformational alteration consistent with the DnaK “remodeling” function or simple blocking of the DnaK 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 TNP (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 TNP 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 aeruginoa 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-
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tion as a proinflammatory mediator by activating the plasma contact activation, or so-called "intrinsich coagulation system (42). The pathology of the pulmonary tularemia sepsis syndrome is characterized by wide dissemination of necrotic foci with histolytic inflammation and pyroganulomas, 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 DnK 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 finally an orchestrated advantage.

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