frontiers in MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 25 April 2011
doi: 10.3389/fmicb.2011.00083

The *capBCA* locus is required for intracellular growth of *Francisella tularensis* LVS

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Francisella tularensis is the causative agent of tularemia and a category A bioterrorism agent. The molecular basis for the extreme virulence of *F. tularensis* remains unclear. Our recent study found that *capBCA*, three neighboring genes, are necessary for the infection of *F. tularensis* live vaccine strain (LVS) in a respiratory infection mouse model. We here show that the *capBCA* genes are necessary for *in vivo* growth of *F. tularensis* LVS in the lungs, spleens, and livers of BALB/c mice. Unmarked deletion of *capBCA* in type A strain Schu S4 resulted in significant attenuation in virulence although the level of the attenuation in Schu S4 was much less profound than in LVS. We further demonstrated that CapB protein is produced at a low level under the *in vitro* culture conditions, and *capB* alone is necessary for *in vivo* growth of *F. tularensis* LVS in the lungs of BALB/c mice. Finally, deletional mutations in *capB* alone or *capBCA* significantly impaired intracellular growth of *F. tularensis* LVS in cultured macrophages, thus suggesting that the *capBCA* genes are necessary for intracellular adaptation of *F. tularensis*. The requirement of this gene locus in intracellular adaption at least in part explains the significant attenuation of *F. tularensis capBCA* mutants in virulence.

Keywords: *Francisella tularensis*, *capBCA*, fitness, virulence, macrophage

INTRODUCTION

Francisella tularensis is a Gram-negative intracellular bacterium and causative agent of tularemia in humans and many other species (Sjostedt, 2007). There are four *F. tularensis* subspecies or biotypes: *tularensis* (type A), *holarctica* (type B), *mediasiatica*, and *novicida*. All *F. tularensis* subspecies are able to cause lethal infection in mice, but only the strains of types A and B are mostly associated with human disease (Keim et al., 2007). *F. tularensis* live vaccine strain (LVS) is a type B derivative. LVS is relatively avirulent in humans but causes a lethal infection in mice that highly resembles human tularemia (Eigelsbach and Downs, 1961; Anthony and Kongshavn, 1987). The infection can be transmitted by inhalation of *F. tularensis*-containing aerosols, bites of blood-sucking insects, handling of infected animal carcasses, and consumption of contaminated food or water. Respiratory tularemia has attracted the most attention because inhalation of as few as 10–50 live organisms of *F. tularensis* type A can cause disease and the mortality rate can be >30% by the respiratory route in the absence of antibiotic therapy (McCrumb, 1961). As a result, *F. tularensis* type A strains are listed as a category A potential agent of bioterrorism (Dennis et al., 2001).

Francisella tularensis is able to infect a range of cell types, but its primary target during infection appears to be the macrophage (Clemens and Horwitz, 2007). The pathogenic mechanisms of *F. tularensis* infection are poorly understood. The extraordinary infectivity of *F. tularensis* is correlated with its efficient uptake, survival, and replication within host cells (Clemens and Horwitz, 2007). The ability of *F. tularensis* to propagate intracellularly is enhanced by the failure of lysosomes to fuse with the phagosome (Anthony et al., 1991) and the lack of stimulation of a respiratory burst (Wilson et al., 1980; Fortier et al., 1994). *F. tularensis* appears to bind host cells by interacting with complement receptors (Clemens et al., 2005). After entry into macrophages, the spacious phagosomes are rapidly modified to a tight phagosome (Clemens et al., 2005). In the initial phase of intracellular infection, *F. tularensis* resides in the membrane-bound phagosomes of macrophages and does not appear to replicate (Golovliov et al., 2003; Clemens et al., 2004; Santic et al., 2005a). Replication occurs once the bacterium escapes into the cytosol when the phagosomal membranes are damaged (Golovliov et al., 2003; Clemens et al., 2004; Santic et al., 2005a). The mechanisms for bacterial escape into the cytosol remain to be defined. It is also unclear how *F. tularensis* disseminates from infected host cells to uninfected cells *in vivo*. It is thought that *F. tularensis*-infected macrophages are lysed by bacterium-induced apoptosis (Lai et al., 2001; Lai and Sjostedt, 2003).

The virulence determinants of *F. tularensis* are not well understood (Barker and Klose, 2007). The lipopolysaccharide (LPS) of *F. tularensis* has been extensively studied because of its unusually low toxicity *in vitro* and *in vivo* and potential as a vaccine component (Sandstrom et al., 1992; Ancuta et al., 1996). Several bacterial proteins have recently been implicated in *F. tularensis* pathogenesis. These include AcpA (Mohapatra et al., 2007), MglA/MglB (Baron and Nano, 1998), and the proteins encoded by the

iglABCD intracellular growth operon (Nano et al., 2004). Recent whole-genome screens in *F. tularensis* strains Schu S4 (Qin and Mann, 2006; Kadzhaev et al., 2009), LVS (Maier et al., 2007; Su et al., 2007; Schulert et al., 2009), and U112 (Tempel et al., 2006; Weiss et al., 2007; Kraemer et al., 2009; Ahlund et al., 2010) have identified a large number of bacterial genes that are involved in *F. tularensis* growth in cultured macrophages and host tissues. Our recent study revealed that the *capBCA* genes of *F. tularensis* strain LVS are necessary for bacterial growth in the lungs of mice (Su et al., 2007). Growth as used here refers to the bacterial load in host tissues, which reflects the net outcome of bacterial replication and survival in the context of host defense.

In this study, we sought to understand how the *capBCA* locus contributes to *F. tularensis* pathogenesis. The *in vivo* growth kinetics of LVS and the isogenic *capBCA* mutants were determined in the lungs, spleens, and livers of infected BALB/c mice at various stages of infection. Unmarked deletion of *capBCA* was generated to assess the significance of this gene locus in the virulence of type A virulent strain Schu S4. We further characterized the expression of *capB* and its impact on *F. tularensis* pathogenesis. Finally, the contribution of *capBCA* to intracellular growth of *F. tularensis* was evaluated in cultured macrophage lines. Our results suggest that the *capBCA* genes contribute to the virulence and pathogenesis of *F. tularensis* at least in part through enhancing bacterial intracellular adaptation.

MATERIALS AND METHODS

BACTERIAL STRAINS AND CHEMICAL REAGENTS

Francisella tularensis LVS and its derivatives were cultured as described previously (Su et al., 2007). When necessary, kanamycin (10 μg/ml) or hygromycin (200 μg/ml) was added in the broth and agar media for selection purposes. *F. tularensis* Schu S4, originally isolated from a human case of tularemia (Eigelsbach et al., 1951), was obtained from the U.S. Army Medical Research Institute for Infectious Diseases (Frederick, MD, USA; Malik et al., 2007). The culture media and conditions for Schu S4 strain are the same as for LVS. *Escherichia coli* strains were grown in Luria-Bertani (LB) broth or on LB agar plates in the presence or absence of ampicillin (100 μg/ml), kanamycin (50 μg/ml), and hygromycin (200 μg/ml). All ingredients for bacterial culture media and other chemicals used in this work were obtained from Sigma (St. Louis, MO, USA) unless otherwise stated.

SITE-DIRECTED MUTAGENESIS IN *F. TULARENSIS*

Unmarked in-frame deletional mutations were generated in the *capBCA* locus of strains LVS and Schu S4 by allelic replacement and counterselection as described (Su et al., 2007). All genetic manipulations of strain Schu S4 were performed with the approval of the Centers for Disease Control and Prevention (CDC) in a CDC-certified ABSL-3/BSL-3 facility at Albany Medical College. The entire coding region of the *capBCA* genes was deleted in Schu S4 using the conjugative plasmid pST937. pST937 was previously used to generate an unmarked deletion in *capBCA* of LVS (Su et al., 2007). *capB* was deleted in LVS in a similar manner with a few modifications. An 814-bp fragment downstream of *capB* was PCR amplified from LVS genomic DNA using primers Pr1073 (5′-TACGAGAATTCTATAGTTTTAAGATTAAACAGGAGAAA-3′) and Pr1074 (5′-CTTGTCTCGAGCATATTTGGATTAACCGAAG ACC-3′). This fragment was digested with *Eco*RI and *Xho*I and cloned behind the 5′ fragment of *capB* in plasmid pST933 (Su et al., 2007). The entire construct was subcloned into the *Eco*RV site of a suicide plasmid pMP590 (LoVullo et al., 2006), resulting in the plasmid pMP590::Δ*capB* or pST968. We chose pMP590 to delete *capB* because of its better amenability in DNA cloning (partially due to its smaller size relative to pomp) and transformation (electroporation instead of conjugation for pDMK). pST968 was transferred into LVS by electroporation and selected for kanamycin resistance (10 μg/ml) as described (LoVullo et al., 2006). To remove the inserted plasmid and generate desirable *capB* deletion, the kanamycin resistant transformants were streaked on chocolate agar plates containing 5% (w/v) sucrose. The sucrose-resistant colonies were further screened for the loss of kanamycin resistance. The resulting clones were examined for the *capB* deletion by PCR amplification using the flanking primers Pr896 (5′-AGCTGCACCTGAGTTATTTGAT-3′) and Pr901 (5′-AAATGCAAATGCGTCGTTA-3′). The *capB* deletion was finally confirmed by DNA sequencing in strain ST1092, one of the resultant Δ*capB* mutants. ST1092 retains the sequence encoding the nine N-terminal amino acids of CapB. The lack of the CapB protein in the Δ*capB* strains was assessed by Western blot using a mouse anti-CapB antiserum as described in western blot. The resultant strain ST1092 was selected for further characterization.

COMPLEMENTATION OF *CAPB* DELETION

The *capB* deletion in strain ST1092 was *in trans* complemented with an *E. coli–Francisella* shuttle plasmid pST1032 containing the intact *capB* gene as described previously (Su et al., 2007). *capB* in pST1032 was driven by the *Francisella groEL*. pST1032 was electroporated into strain ST1092 and selected with 200 μg/ml hygromycin, resulting in strain ST1104. Production of the CapB protein in the complemented strains was assessed by western blot using a mouse antiserum against glutathione *S* transferase (GST)-CapB as described in Section "Antibodies and Western blotting."

GST-CAPB FUSION PROTEIN EXPRESSION

A recombinant CapB was expressed as a fusion protein with the GST essentially as described (Lu et al., 2006). *capB* was amplified from the genomic DNA of strain LVS with primers Pr885 (5′-ATCCTGAATTCGGATCCATATTTTCTCCTGTTT-3′) and Pr1175 (5′-ACTAGACCCGGGAACTACTTTGGATTTTTGGTTA ATTG-3′). This fragment was cloned into the *Xma*I/*Eco*RI site of the pGEX-2T expression vector (GE Healthcare Bio-Science, Piscataway, NJ, USA) in *E. coli* strain BL-21 (DE3). The resultant *E. coli* strain was processed to produce and purify a GST fusion protein (designated GST-CapB) by affinity chromatography with the Glutathione Sepharose 4 Fast Flow resins (GE Healthcare Bio-Sciences) according to the supplier's instructions. Protein concentration was determined by the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA, USA) and analyzed by SDS-PAGE as described (Lu et al., 2006).

ANTIBODIES AND WESTERN BLOTTING

Antiserum against the *F. tularensis* CapB was raised with the GST-CapB fusion protein as described (Zhang et al., 1997). Female BALB/c mice (6–8 weeks old) were immunized with purified

GST-CapB every 2 weeks via the subcutaneous route for a total of three immunizations. The immunogen for each immunization consisted of 25 μg GST-CapB in 100 μl sterile phosphate-buffer saline (PBS) and 100 μl alum (Rehydrogel Low Viscosity Gel; Reheis, Berkeley Heights, NJ, USA) as described (Sun et al., 2004). Western blotting of *F. tularensis* proteins was performed with an enhanced chemiluminescence (ECL) Western Blot Kit (Pierce, Rockford, IL, USA) according to the supplier's instructions. The GST-CapB antiserum and peroxidase-conjugated goat anti-mouse IgG antibody (Bio-Rad laboratories, Hercules, CA, USA) were used at a dilution of 1:1000 and 1:5000, respectively.

MOUSE INFECTIONS

Infection experiments with *F. tularensis* LVS and its derivates were carried out in BALB/c mice as described (Su et al., 2007). All animal infection experiments were in compliance with the guidelines of the Institutional Animal Care and Use Committee at Albany Medical College. To prepare the *F. tularensis* inocula, frozen stocks of LVS and the isogenic *capBCA* mutants were individually diluted in PBS based on predetermined colony forming unit (CFU) values. Each preparation was serially diluted in PBS and plated to assess the actual CFUs of each inoculum immediately prior to the mouse infection experiments. Each inoculum was intranasally inoculated into groups of mice (female, 6–8 weeks old). Infected mice were sacrificed at various time points post-infection. The lungs, liver, and spleen were aseptically removed and processed to determine the CFU levels of LVS and the mutants in each organ as described (Su et al., 2007). To determine the virulence levels of the Δ*capBCA* mutants in the LVS and Schu S4 backgrounds, groups of five mice were intranasally infected with serial dilutions of the wild type and isogenic Δ*capBCA* strains. Infected mice were monitored daily for signs of morbidity and mortality for 21 days. Infection experiments with Schu S4 and all of its derivatives were carried out as described for strain LVS with the exception that all of the experiments associated with Schu S4 were performed and contained in a CDC-certified ABSL-3/BSL-3 facility at Albany Medical College.

To test the immuno-protection of Δ*capBCA* mutants, groups of five BALB/c mice were inoculated intranasally with different dosages of ST938. Mice injected with sterile PBS were used as control. Three weeks after immunization, each mouse was challenged intranasally with 40,000 CFU of LVS. Infected mice were monitored daily for clinical signs and mortality for 21 days.

HISTOPATHOLOGY

BALB/c mice were intranasally infected with *F. tularensis* LVS or isogenic mutants as described above. Mice, along with uninfected controls, were sacrificed to excise the lungs, liver, and spleen at day 7. The organs were fixed in 10% neutral-buffered formalin, processed using standard histological methods to obtain 5-μm-thick paraffin sections, and stained with H&E as described (Baron et al., 2007).

MACROPHAGE INFECTION

The human monocytic cell line U937 (ATCC CRL-1593.2) and mouse alveolar macrophages MH-S cell line (ATCC CRL-2019) were obtained from the American Type Culture Collection (Manassas, VA, USA). U937 cells were maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal bovine serum at 37°C, 5% CO_2; MH-S cells were cultured in RPMI 1640 medium containing 10% (v/v) fetal bovine serum and 0.05 mM 2-mercaptoethanol at 37°C. Intracellular levels of *F. tularensis* were assessed out as described (Santic et al., 2005b). Briefly, 2.5×10^5 macrophages were cultured in 24-well plates (Hyclone, Logan, UT, USA) to approximately 80% confluency. The *F. tularensis* LVS derivatives were grown in MHB broth to OD_{600} ~ 1.2 (~3×10^9 CFUs/ml), pelleted by centrifugation, and resuspended in pre-warmed RPMI1640 to OD_{600} ~ 0.2 (~5×10^8 CFUs/ml). After brief rinse with PBS, macrophages were infected with the bacterial suspensions at a multiplicity of infection (MOI) of 100 (bacterium vs. macrophage) at 0 h. After 2 h of incubation at 37°C, the unbound bacteria were removed from the wells by washing with PBS. Extracellular bacteria were killed by incubation with the cell culture medium containing gentamicin (50 μg/ml) for 1 h at 37°C. The cells were extensively rinsed to remove residual gentamicin and then cultured without antibiotic. To monitor bacterial uptake and replication, the culture medium of infected macrophages was moved to sterile tubes to lyse the cells at 3, 24, and 48 h post-infection with pre-chilled water. The medium and lysates from each well were combined, diluted with sterile PBS, and spread onto chocolate agar plates for CFU counts. Each infection experiment was repeated at least three times.

FLUORESCENCE AND ELECTRON MICROSCOPY

For fluorescence microscopy, U937 macrophages were infected with *F. tularensis* LVS as described previously (Santic et al., 2005a). Briefly, differentiated U937 cells on 12-mm-diameter circular glass coverslips (Fisher Scientific, Pittsburgh, PA, USA) in 24-well culture plates were infected with LVS and its isogenic mutants at MOI of 10 for 1 h. To synchronize the infection, infected cells were centrifuged at $150 \times g$ for 5 min before incubation at 37°C in 5% CO_2. After 1 h of incubation, infected cells were washed three times with 1× PBS followed by 1 h of gentamicin (50 μg/ml) treatment. Cells were then fixed in 4% paraformaldehyde for 30 min. Paraformaldehyde was removed by washing the wells three times with PBS. The infected cells were permeabilized with 1% Triton-X100 for the LAMP-1 experiment. For the Cathepsin D experiments, the cells were permeabilized with acetone for 5 min at −20°C. Samples were stained with *F. tularensis* polyclonal antibody (1:4,000 dilution) and mouse monoclonal anti-LAMP-1 (H4B3; 1:500 dilution; Hybridoma Gene Bank) or anti-Cathepsin D (BD transduction; 1:500 dilution). Alexa fluor 594-conjugated anti-mouse secondary antibody (1:500 dilution) was used to stain the LAMP-1 and Cathepsin D. Alexa fluor 488-conjugated secondary antibody (1:500 dilution) was used to stain *F. tularensis.* Co-localization of bacteria with LAMP-1 and Cathepsin D was analyzed with FV1000 Olympus confocal microscope as described previously (Santic et al., 2005a,b, 2008). The bacteria associated with phagosomes and phagolysosomes were quantified by enumerating *F. tularensis* co-localized with LAMP-1/Cathepsin D out of the total bacteria in 100 infected cells from more than 10 different fields for each strain. Each experiment was repeated at least once.

For electron microscopy, U937 macrophages growing in 6-well tissue culture plates were inoculated with *F. tularensis* as described above. Twenty-four hours post-inoculation, the wells were washed with 0.1 M Na Cacodylate buffer, pH 7.3 and incubated for 1 h with

a solution containing 2.5% glutaraldehyde/4% formaldehyde in 0.1 M Na Cacodylate, pH 7.3. Following washing with Cacodylate buffer, the cells were post-fixed with 1.0% osmium tetroxide in 0.1 M Na Cacodylate buffer, pH 7.3 for 1 h. The monolayers were washed with normal saline, and scraped with a Costar cell scraper into normal saline and gently pelleted by centrifugation. The pellets were dehydrated in a graded Ethanol series and embedded in LR White medium (Polysciences, Inc.). Ultrathin sections were collected onto Formvar-coated grids, stained with uranyl acetate and lead citrate. Specimens were viewed and photographed with a JEOL 100CX transmission electron microscope using Kodak electron microscope film.

STATISTICAL ANALYSIS

A log-rank test was used to determine the level of significance for the Kaplan–Meier survival analyses. All other results were expressed as mean ± SEM and comparisons between the groups were made using Student's *t* test. Differences between control and experimental groups were considered significant at $p < 0.05$ levels.

RESULTS

IN VIVO GROWTH KINETICS OF THE LVS *capBCA* MUTANTS

In our previous study (Su et al., 2007), the LVS mutant lacking the *capBCA* genes (Δ*capBCA*) exhibited significantly impaired growth in the lungs of infected mice at day 7 post-intranasal inoculation. However, it was unclear whether the *capBCA* genes are required at the earlier and/or later stages of *F. tularensis* infection. We thus determined the *in vivo* growth kinetics of Δ*capBCA* by assessing the levels of bacterial burdens in the lungs, kidneys, and livers of infected BALB/c mice on days 1, 2, 4, 7, 14, and 21 post-intranasal inoculation.

To accurately determine the inoculation efficiency, we first determined bacterial loads in the lungs of infected mice immediately after intranasal inoculation. Mice infected with LVS or Δ*capBCA* (strain ST938) showed a similar level of bacteria (~3×10^3 CFUs) in the lungs 1 h post-intranasal inoculation of 5×0^3 CFUs (day 0; **Figure 1A**). This indicated that Δ*capBCA* was effectively inoculated into the lungs of mice at a similar efficiency as the parent strain LVS. Δ*capBCA* displayed a significant growth deficiency in the lungs throughout the entire test period (**Figure 1A**). In sharp contrast to a 15.1-fold growth of LVS in the lungs of infected mice in initial 24 h, the number of Δ*capBCA* dropped by 75% during the same infection period, indicating that the *capBCA* locus is required for bacterial growth in the initial phase of LVS infection. At days 2 and 4, both LVS and Δ*capBCA* exhibited substantial growth, but the numbers of Δ*capBCA* were significantly lower when compared with that of LVS. At day 7, the bacterial levels of both LVS and Δ*capBCA* were reduced bacterial burdens, likely due to the onset of the adaptive immunity. By 9 days post-inoculation, all of the LVS-infected mice died, whereas Δ*capBCA*-infected mice did not display any detectable sign of disease and remained disease-free throughout the 21-day infection period. Finally, Δ*capBCA* became undetectable from the lungs of the infected mice at days 14 and 21.

We also assessed the dissemination of Δ*capBCA* from lung to spleen and liver in the same BALB/c mice as described in **Figure 1A**. As early as 24 h post-inoculation, LVS were readily detected in the spleens (2,430 CFUs in average) and livers (42,840 CFUs in average), whereas Δ*capBCA* was barely detectable in these distal organs until day 4 (**Figures 1B,C**). Similar to the kinetics of bacterial burden in the lungs, the numbers of Δ*capBCA* were significantly lower than those of LVS in the spleens and livers from days 1 to 7. Δ*capBCA* became undetectable in the livers of all infected mice on days 14 and 21. Interestingly, there were low levels of Δ*capBCA* detectable in the spleens of two and one mice (out of six) at days 14 and 21, respectively (see below for additional interpretations). We thus conclude that the *capBCA* genes are necessary for *in vivo* growth of *F. tularensis* LVS (bacterial replication and resistance to host killing).

LD_{50} AND IMMUNO-PROTECTION OF LVS Δ*capBCA*

The persistent presence of Δ*capBCA* in the spleens (**Figure 1B**) raised the possibility that the mutant is capable of better priming the adaptive immunity against *F. tularensis* LVS. To test this notion, we first attempted to assess the attenuation level of Δ*capBCA* by determining the LD_{50}. Groups of BALB/c mice were intranasally infected with a wide range of bacterial doses ($4.5 \times 10^{2-7}$ CFUs). None of the infected mice showed any appreciable symptoms or died from the infection during the 6-week period of observation (data not shown), indicating the LD_{50} of the Δ*capBCA* mutant

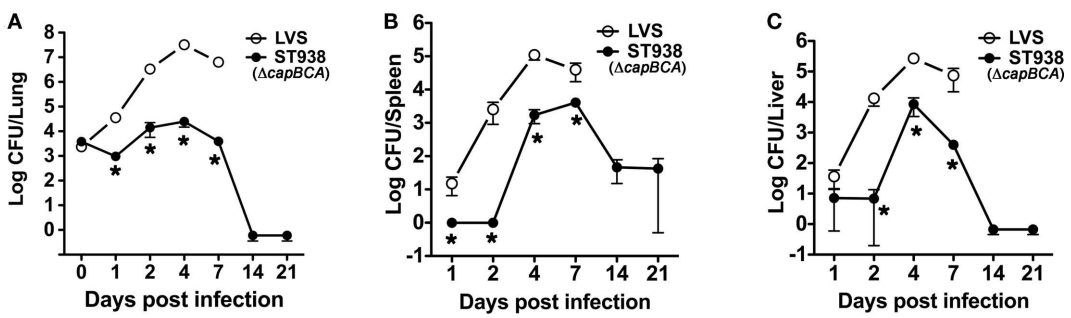

FIGURE 1 | *In vivo* growth of LVS Δ*capBCA*. (A) Bacterial burden in the lungs of BALB/c mice ($n = 5$) infected with LVS or isogenic Δ*capBCA* strain ST938 alone. Lung tissues were collected to determine the viable bacteria by plating various dilutions of tissue homogenates at days 0, 1, 2, 4, 7, 14, and 21 post-inoculation. Bars indicate the mean $\log_{10}$CFU values ± SEM. Asterisks indicate $p < 0.05$ as determined by Student's *t* test. **(B)** Bacterial burdens in the spleens of mice infected individually with LVS derivatives were determined as in **(A)**. **(C)** Bacterial burdens in the livers of mice infected individually with LVS derivatives were determined as in **(A)**.

was >4.5 × 10^7 CFU. LVS has an LD_{50} of ~4 × 10^3 CFUs in this infection model (Duckett et al., 2005); all of mice infected with ~8 × 10^3 CFUs of LVS died in the first 2 weeks of infection (data not shown). Therefore, LVS Δ*apBCA* is avirulent in this BALB/c mouse model.

We next tested immuno-protection of Δ*capBCA* against infection of *F. tularensis* LVS. Groups of five BALB/c mice were intranasally infected (or immunized) with ST938 in a dose range of 4.5 × $10^{2–7}$ CFUs. The mice were intranasally challenged with 40,000 CFUs of LVS (10 times of LVS LD_{50} in this model) 3 weeks post-immunization. All of the five unimmunized mice (negative control) died within 9 days following LVS challenge (**Table 1**). Similarly, the majority (80%) of the mice immunized with 450 CFUs of ST938 succumbed to LVS challenge. In contrast, all of the mice immunized with higher doses of ST938 (4.5 × $10^{3–7}$ CFUs) did not show appreciable sign of illness after the challenge with 40,000 CFUs of LVS in the 4-week observation period (**Table 1**), indicating that Δ*capBCA* is able to induce strong adaptive immunity against infection of *F. tularensis* LVS even at relative low doses of immunization (e.g., $10^{3–4}$ CFUs). This result agrees with a recent report that an LVS Δ*capB* strain induces potent immunoprotection against LVS challenge (Jia et al., 2010). We further tested the immuno-protection potential of Δ*capBCA* against the challenges with type A virulent strain Schu S4 in a similar manner. None of the intranasal immunization doses (4.5 × $10^{3–7}$ CFUs of strain ST938) conferred significant protection against intranasal challenge with 100 CFUs of Schu S4 (data not shown). The sharp difference in immuno-protection of LVS Δ*capBCA* against LVS and Schu S4 demonstrate the distinct requirements for protective immunity against infections of types A and B *F. tularensis*.

IMPORTANCE OF *capBCA* IN TYPE A *F. TULARENSIS*

The gene sequence and organization of the *capBCA* locus are virtually identical between *F. tularensis* subsp. *holarctica* (type B) and subsp. *tularensis* (type A). We thus tested whether the *capBCA* genes are necessary for the virulence of *F. tularensis* type A virulent strain Schu S4. The coding sequence of the *capBCA* locus in Schu S4 was deleted by allelic replacement and counter selection as described in Section "Materials and Methods." As observed with the Δ*capBCA* mutants in LVS (Su et al., 2007), the Schu S4 Δ*capBCA* mutants did not show obvious growth defects under *in vitro* conditions (data not shown). ST965, one of the Schu S4 Δ*capBCA* mutants, was subjected to further characterization of its virulence in the lung infection mouse model. Since laboratory mice are extremely susceptible to Schu S4 infection (Malik et al., 2007), the infection doses of the Δ*capBCA* mutant were accordingly adjusted. While all of mice infected with 16 CFUs of Schu S4 died by day 9, the mortality rate of mice infected with ST965 was significantly reduced in a dose-dependent manner (**Figure 2**). In general, the attenuation of the Schu S4 Δ*capBCA* mutant was less pronounced than that of the LVS counterpart in this model. Michell et al. (2010) recently reported that deletion of *capB* in Schu S4 led to >100-fold attenuation in virulence in a subcutaneous infection model of BALB/c mice. These findings thus indicated that the *capBCA* locus is necessary for the full virulence of human virulent *F. tularensis* type A strains.

Table 1 | Protection of LVS Δ*capBCA* against lethal LVS infection.

Immunization dose (CFU)	Survived/total mice
0	0/5
4.5 × 10^2	1/5
4.5 × 10^3	5/5
4.5 × 10^4	5/5
4.5 × 10^5	5/5
4.5 × 10^6	5/5
4.5 × 10^7	5/5

CHARACTERIZATION OF *F. TULARENSIS capB*

To gain more insight into the function(s) of the *F. tularensis capBCA* locus, we further characterized *capB*. The coding sequence of *capB* in LVS was removed by allelic exchange. Three resulting strains (ST1092-4) were verified for the lack of *capB* as determined by PCR amplification (**Figure 3A**) and DNA sequencing (data not shown). Primers Pr896/Pr901 were expected to produce amplicons of 2,177 bp in LVS and 991 bp in Δ*capB* mutants ST1092-4. To determine the expression of the CapB protein, we also constructed a GST-CapB fusion protein in *E. coli* to generate an antiserum against CapB in mice. The *F. tularensis* CapB protein is predicted to contain 405 amino acids with the molecular mass of 44.9 kDa. As represented in **Figure 3B**, the antiserum reacted with a protein band of 44 kDa in LVS, which was completely absent in the isogenic Δ*capB* mutant ST1092. The missing band in ST1092 was restored *in trans* by the shuttle plasmid pST1032 (strain ST1104). We thus concluded that the antiserum specifically detected the CapB protein. The CapB protein appears to be produced at a relatively low level under *in vitro* culture conditions because it was only detected when a large amount of the total bacterial lysates (~2 × 10^9 CFU) was loaded in the protein gel. This was not due to a potential low titer of the antiserum because the antiserum readily detected CapB with ~2 × 10^8 CFUs of the complementation strain ST1104 (data not shown). Apparent overproduction of CapB in ST1104 was likely due to the strength of the *Francisella groEL* promoter and/or copy number of the

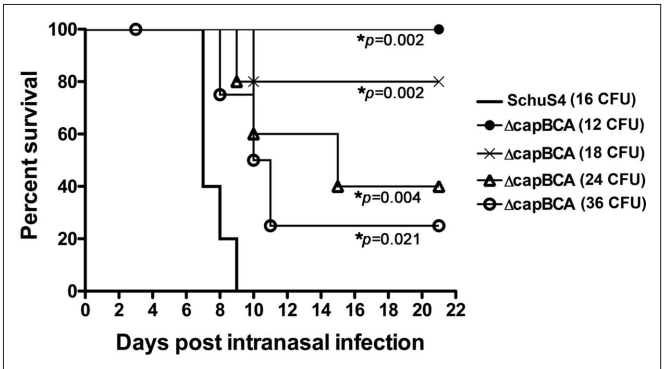

FIGURE 2 | Contribution of *capBCA* to the virulence of type A *F. tularensis*. BALB/c mice (n = 5) were intranasally infected with the indicated doses of Schu S4 or isogenic Δ*capBCA* mutant ST965. Survival was monitored for 21 days. The results are expressed as Kaplan–Meier curves and p values determined using log-rank test. The data shown are a representative of two independent experiments.

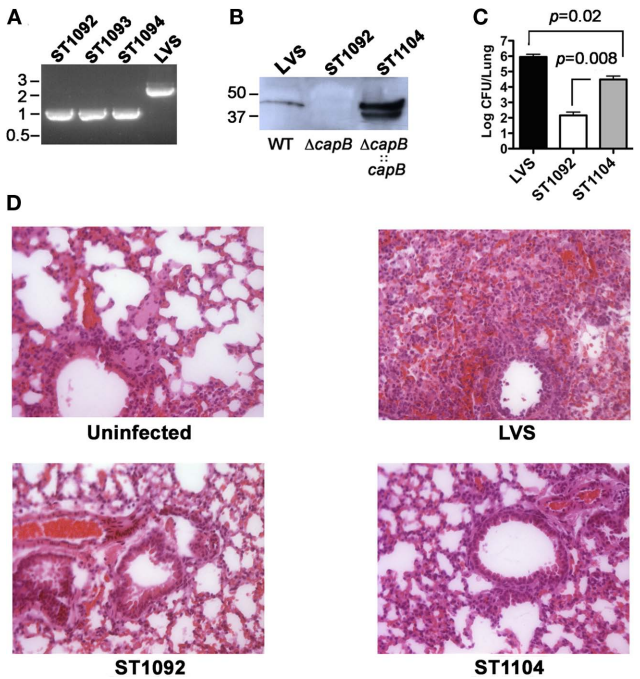

FIGURE 3 | CapB expression and its impact on *in vivo* growth of *F. tularensis*. (A) Detection of the *capB* deletion by PCR. The sequence surrounding the *capB* gene was amplified from the genomic DNA of LVS or three isogenic Δ*capB* clones (ST1092-4). The PCR products were separated in a 1% agarose gel and stained with ethidium bromide. The sizes of the DNA standards are indicated in kilobase. **(B)** Western blot detection of the *F. tularensis* CapB. Cell lysates of LVS derivatives representing ~2×10^9 CFUs were separated by SDS-PAGE, probed with the GST-CapB antiserum, and detected by an ECL method. **(C)** *In vivo* growth of the *F. tularensis* Δ*capB* mutant. BALB/c mice were infected individually with LVS (4,500 CFUs), ST1092 (5,100 CFUs), or ST1104 (4,800 CFUs) by intranasal inoculation. Bacterial levels in the lungs were assessed at day 7 as in **Figure 1**. **(D)** Lung histopathology of *F. tularensis*-infected mice. BALB/c mice were infected as in **(C)**. Lung tissues were processed for H&E staining at day 7 post-infection. Magnification = 20×.

complementation construct as reported by Charity et al. (2007). A second band detected by the antiserum in ST1104 may represent a smaller isoform of the *F. tularensis* CapB as reported for the *B. anthracis* CapB (Makino et al., 1989).

We further performed infection experiments with the Δ*capB* mutant ST1092 and isogenic complementation strain ST1104. The Δ*capB* mutant was significantly deficient in *in vivo* growth at day 7 post-intranasal inoculation compared to the parent strain LVS (**Figure 3C**; **Figure A1** in Appendix). The attenuation phenotype of ST1092 was partially *in trans* restored in complementation strain ST1104. The same *capB* complementation construct also showed partial complementation in the transposon mutant JS2512 in our previous study (Su et al., 2007). This could be due to instability of the shuttle plasmid during infection in the absence of antibiotic selection and/or inappropriate *in trans* expression level of the CapB protein. We also examined the impact of *capB* on *F. tularensis* pathogenesis by comparing histopathology of the lungs from BALB/c mice intranasally infected with either LVS or isogenic Δ*capB* mutants. In agreement with previous studies (Baron et al., 2007; Malik et al., 2007), the lungs of the LVS-infected mice had severe inflammation and tissue damage at day 7 post-infection as compared to the uninfected control (**Figure 3D**). In contrast, mice infected with ST1092 showed a relatively normal lung structure with little sign of inflammation. The lungs of mice infected with the *capB* complementation strain ST1104 exhibited a low level of tissue infiltration but the overall structure was relatively normal. Together with the experimental data with Δ*capBCA* (**Figure 1**; Su et al., 2007), these results allowed us to conclude that *capB* itself is necessary for the fitness of *F. tularensis* LVS. Our data are also consistent with a very recent study reporting that an LVS Δ*capB* mutant is severely attenuated in BALB/c mice (Jia et al., 2010).

DEFICIENCY OF THE *capBCA* MUTANTS IN INTRA-MACROPHAGE GROWTH

We sought to understand how the *capBCA* locus contributes to *in vivo* adaptation of *F. tularensis*. Since *F. tularensis* is a facultative intracellular pathogen, we tested whether the *capBCA* genes are required for intracellular growth of *F. tularensis*. We initially tested the intracellular infection of Δ*capB* and Δ*capBCA* in mouse MH-S macrophages, because this is a commonly used alveolar macrophage model derived from BALB/c mice (Mbawuike and Herscowitz, 1989; Ibrahim-Granet et al., 2003). At the end of the gentamicin treatment (hour 0), Δ*capB* and Δ*capBCA* showed similar levels of the intracellular growth as the parent strain LVS (**Figure 4A**). Because *F. tularensis* does not appear to replicate in the first 3 h post-infection (Golovliov et al., 2003; Clemens et al., 2004; Santic et al., 2005a), the result indicated that *capBCA* locus is dispensable for the initial phase of intracellular infection (bacterial adherence and uptake). By 24 and 48 h post-inoculation, the macrophages infected with Δ*capB* or Δ*capBCA* showed significantly lower CFUs (approximately 14- to 22-fold reduction) as compared to the cells infected with LVS (**Figure 4A**). Noticeably, Δ*capB* and Δ*capBCA* behaved similarly in this assay, suggesting that *capB* carries out an important function(s) in this locus. The growth defect of Δ*capB* was not due to the abnormal growth of the mutant in the culture medium because LVS and its *capBCA* derivatives showed obvious growth in the absence of macrophages under the same conditions (data not shown). The defect of Δ*capB* in intracellular growth was partially restored by *in trans* complementation (**Figure 4A**, Δ*capB::capB*). The complementation strain still exhibited significant deficiency (two- to threefold) in intracellular growth as compared to LVS, likely due to overexpression of *capB* on the complementation construct (see **Figure 3B**). We were unsuccessful in our attempts to complement the entire *capBCA* locus due to technical difficulty in cloning the three genes in *E. coli–Francisella* shuttle vectors (data not shown).

We subsequently confirmed the impairment of Δ*capB* and Δ*capBCA* in intracellular growth using human U937 macrophages. Like in MH-S cells, the intracellular growth defect of Δ*capB* and Δ*capBCA* was only observed after the initial phase of infection (**Figure 4B**, hour 0). A noticeable difference between the MH-S and U937 results is the marginal effect of *in trans* complementation construct on the intracellular growth phenotype of Δ*capB* in U937 cells (**Figure 4B**, Δ*capB::capB*). A very recent study by Jia et al. (2010) also showed that an LVS Δ*capB* mutant is significantly

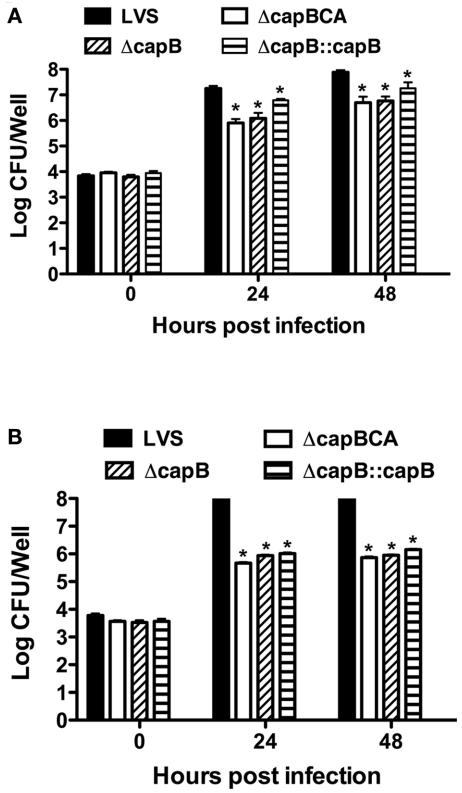

FIGURE 4 | Intracellular growth of *F. tularensis capBCA* mutants. **(A)** Intracellular growth of LVS derivatives in MH-S macrophages. Macrophages were infected at a multiplicity of infection of ~100:1 (bacterium vs. macrophage) with LVS, isogenic Δ*capBCA* (strain ST938), Δ*capB* (strain ST1092), or complemented Δ*capB* (Δ*capB::*capB, strain ST1104). Total bacteria per well were enumerated at the indicated time points. Statistic significance between LVS and its isogenic *capBCA* mutants ($p > 0.05$) was determined using Student's *t* test. **(B)** Intracellular growth of LVS derivatives in U937 macrophages. The infection experiments were carried out and analyzed as in **(A)**.

attenuated in intracellular growth in THP-1 macrophages. Together, these results demonstrate that the *capBCA* locus is required for intracellular growth of *F. tularensis* LVS.

SUB-CELLULAR LOCALIZATION OF THE *capBCA* MUTANTS IN MACROPHAGES

Previous studies indicate that *F. tularensis* transiently resides in a LAMP-1 and LAMP-2 positive phagosomes before escape into the cytosol for replication (Santic et al., 2010). The existing data have shown a correlation between escape into the cytosol and loss of LAMP-1/2 co-localization (Checroun et al., 2006; Bonquist et al., 2008; Asare and Abu Kwaik, 2010). Therefore, loss of co-localization with LAMP-1 and LAMP-2 has been used as an indicator for cytosolic localization of *F. tularensis* (Buchan et al., 2009). Based on the importance of the *capBCA* genes in *F. tularensis* intracellular growth (**Figure 4**), we reasoned that this locus might play a role in the phagosomal escape of the bacterium and/or arrest of phagosomal maturation. To test this possibility, we analyzed co-localization of Δ*capB* and Δ*capBCA* with LAMP-1 (a phagosomal marker) and Cathepsin D (a lysosomal marker) in U937 macrophages by immunofluorescence microscopy.

As exemplified in **Figures 5A,C**, only a small fraction (31%) of wild type LVS were associated with LAMP-1 and the majority of LVS bacteria were found in cytosol by 2 h post-infection, suggesting successful escape of LVS from the phagosome to cytosol. In sharp contrast, the majority of Δ*capB* (61%) and Δ*capBCA* (72%) were found to be associated with LAMP-1 during the same infection. These LAMP-1 co-localization levels are close to that of inactivated LVS (73%). Similar to the intracellular growth data (**Figure 4B**), *in trans* complementation of Δ*capB* with the wild type gene on a shuttle plasmid had marginal effect on the LAMP-1 co-localization of the mutant (**Figure 5C**). This finding thus suggested that Δ*capB* and Δ*capBCA* were severely impaired in their ability to escape from the phagosomes into cytosol.

Consistent with the co-localization of both the mutants with LAMP-1, Δ*capB* (68%) and Δ*capBCA* (73%) predominantly co-localized with Cathepsin D, a lysosomal marker, by 2 h post-infection (**Figures 5B,C**). As a positive control, only 27% of live LVS was co-localized with Cathepsin D, which is consistent with its ability to escape from the phagosome–lysosome fusion pathway. In contrast, the inactivated LVS was mostly associated with Cathepsin D (83%) in the same infection period. Similar to the result for LAMP-1 (**Figure 5A**), the *capB* complementation construct failed to restore the co-localization of Δ*capB* to the level exhibited by the wild type bacteria (**Figures 5B,C**). We subsequently confirmed these findings in a Δ*capB* mutant of *F. novicida* strain U112 (data not shown). These results strongly suggested that Δ*capB* and Δ*capBCA* are significantly impaired in their ability to arrest maturation of the phagosome.

Finally, we visualized Δ*capB* and Δ*capBCA*, and their sub-cellular localization in U937 macrophages by transmission electron microscopy. We focused our analysis on the 24-h time point because the morphology of the infected macrophages was no longer intact beyond this infection period. As represented in **Figure 6** (indicated by arrow heads), the LVS-infected cells contained numerous bacteria that appear to be free in the cytoplasm and surrounded by an electron lucent zone. However, in addition to the free bacteria, there were large numbers of partially degraded bacteria in vacuolar-like structures in the macrophages infected with Δ*capB* (strain ST1092) and Δ*capBCA* (strain ST938) as indicated by asterisks in **Figure 6**. This type of sub-cellular structures were rarely found in the LVS-infected macrophages (data not shown). The structures containing apparently degraded bacteria were also readily detectable in the *in trans* complemented Δ*capB* (strain ST1104), indicating that the complementation construct failed to restore the impairment in Δ*capB*. These bacterium-containing vacuolar-like structures are likely phagolysosomes or autophagosomes because most of them were surrounded by an identifiable membrane. Taken together, we conclude that the *capBCA* genes are required for *F. tularensis* phagosomal escape and/or arrest of phagosomal maturation.

DISCUSSION

The *F. tularensis capBCA* genes are among the 95 virulence-associated genes identified in our recent STM study (Su et al., 2007). Transposon insertions in each of the *capBCA* genes resulted in significantly impaired growth of *F. tularensis* LVS in the lungs of BALB/c mice 7 days post-intranasal inoculation. In this study, we sought to understand how this gene locus contributes to

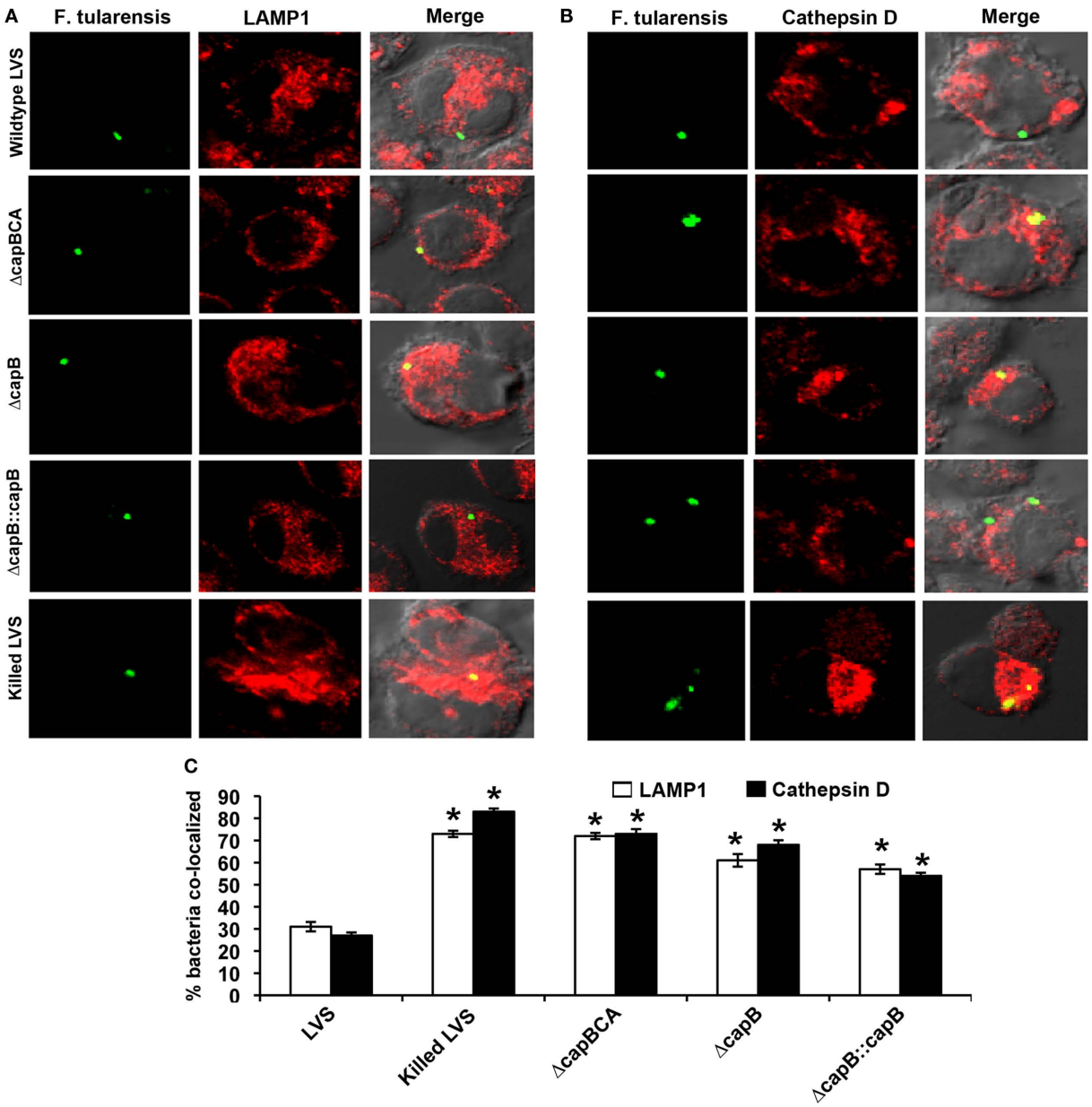

FIGURE 5 | Co-localization of *F. tularensis capBCA* mutants with phagosomal and phagolysosomal markers. (A) Representative images showing co-localization of the LVS *capBCA* mutants with the phagosomal marker LAMP-1. *F. tularensis* and LAMP-1 were stained and visualized as described in Section "Materials and Methods." **(B)** Representative images showing co-localization of the LVS *capBCA* mutants with phagolysosomal marker Cathepsin D. **(C)** Association of LVS *capBCA* mutants with LAMP-1 and Cathepsin D. The results are expressed as the percentage of bacteria co-localized with LAMP-1 or Cathepsin D out of total bacteria in 100 U937 cells. Statistic significance between LVS and its isogenic *capBCA* mutants ($p > 0.05$) was determined using Student's *t* test. The data shown are a representative of two independent experiments.

F. tularensis fitness and pathogenesis. Mouse infection experiments demonstrated that the unknown function(s) provided by the *capBCA* genes is required for the fitness and virulence of *F. tularensis* LVS and human virulent type A strain Schu S4. This finding is consistent with the recent reports that deletion mutants in *capB* of LVS (Jia et al., 2010) and Schu S4 (Michell et al., 2010) are attenuated in BALB/c mice. The experiments with the LVS *capBCA* mutants indicated that the *capBCA* genes are necessary for *F. tularensis* growth (replication and survival) in target organs at various stages of infection. The results from macrophage infection experiments suggest that the *capBCA* genes enhance *F. tularensis* fitness and thus virulence by promoting intracellular growth of the bacterium. This conclusion is supported by significant deficiency of Δ*capB* and Δ*capBCA* in intracellular growth in both MH-S and U937 macrophage models.

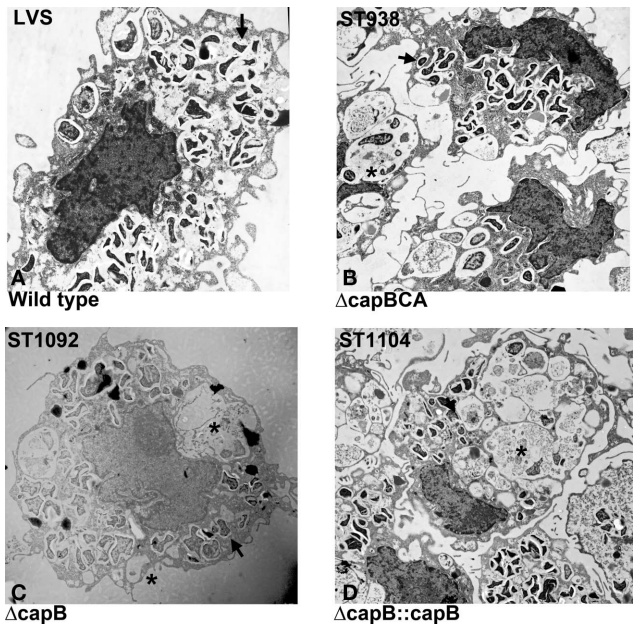

FIGURE 6 | Sub-cellular ultra-structures of U937 macrophages infected by LVS *capBCA* mutants. Electron micrographs of human macrophages inoculated with *F. tularensis* LVS **(A)**, Δ*capBCA* (ST938) **(B)**, Δ*capB* (ST1092) **(C)**, or complemented Δ*capB* (ST1104) **(D)**. Indicated are the intact bacteria (arrow heads) and vacuoles containing degraded bacteria (asterisks).

The deficiency of the *capBCA* mutants in intracellular growth lies beyond the uptake (attachment/entry) phase of intracellular infection. Specifically, the *capBCA* locus appears to enhance the intracellular growth of *F. tularensis* by promoting bacterial escape from phagosomes. This notion is consistent with multiple observations in this study. Δ*capB* and Δ*capBCA* remained predominantly co-localized with the LAMP-1 (a phagosomal marker) and Cathepsin D (a phagolysosomal marker) in U937 macrophages by 2 h post-infection. Second, degraded bacteria were abundantly observed in vacuolar-like structures of the macrophages infected with Δ*capB* and Δ*capBCA*. In this regard, the *capBCA* mutants behaved similarly to the mutants of other *F. tularensis* genes that are involved in phagosomal escape, most notably, the *iglABCD* genes located within the *Francisella* pathogenicity island (FPI; Lai et al., 2004; Nano et al., 2004; Santic et al., 2005b, 2008; de Bruin et al., 2007). However, we noticed that the *capBCA* mutants were much less impaired than the mutants of *iglC* or *mglA* (encoding a FPI regulator) in terms of intra-macrophage growth (data not shown). While the *iglC* or *mglA* mutants virtually lacked intra-macrophage growth, the *capBCA* mutants were still able to grow to a relatively high level under the same conditions (data not shown). This observation indicates that the *capBCA* and FPI genes perform distinct functions in terms of promoting intracellular adaptation of *F. tularensis*. In short, it remains to be determined whether the *capBCA* mutants are defective in one or more of the following intracellular infection stages: (i) survival/escape from the phagosomes to the cytosol, (ii) arrest of phagosomal maturation, (iii) replication in the cytosol, and/or (iv) re-infection of other cells.

It is unclear how the *capBCA* genes contribute to *F. tularensis* growth in macrophages and host tissues. *F. tularensis* CapB and CapC are homologous to the CapB and CapC proteins of *Bacillus anthracis*, the causative agent of anthrax (Koehler, 2002). CapB and CapC, together with CapA, CapD, and CapE in *B. anthracis*, are responsible for the biosynthesis of the capsule consisting of poly-γ-D-glutamic acids (PGA; Candela and Fouet, 2006). CapB, cytoplasmic protein, is the catalytic component of the *Bacillus* PGA synthesis complex (Troy, 1973). The PGA-based capsule in *B. anthracis* is a major virulence factor due to its antiphagocytic property (Koehler, 2002). Interestingly, recent genome studies have revealed homologs of the PGA biosynthetic genes in a group of highly diverse Gram-negative bacteria including *F. tularensis* and *Fusobacterium nucleatum* (Kapatral et al., 2002; Glockner et al., 2003; Ren et al., 2003; Hou et al., 2004; Larsson et al., 2005). Candela et al. (2009) recently reported the production of PGA by the *capBCA* genes of *F. nucleatum*. The *capBCA* loci of *F. tularensis* and *F. nucleatum* are highly similar in gene sequence and order. It is thus possible that the *F. tularensis capBCA* locus enhances the bacterial growth in macrophages and host tissues through PGA production. However, no PGA or capsule has been detected from *F. tularensis* in our preliminary trials (unpublished data) or previous studies (Raynaud et al., 2007; Michell et al., 2010).

The *capBCA* locus is highly conserved in genus *Francisella*. The sequences of the *capBCA* coding and intergenic regions are virtually identical among *F. tularensis* subsp. *tularensis* (type A) (Larsson et al., 2005; Beckstrom-Sternberg et al., 2007), *holarctica* (type B) (Chain et al., 2006; Petrosino et al., 2006; Barabote et al., 2009), and *novicida* (Brittnacher et al., 2007). A recent study also revealed the presence of a similar *capBCA* locus in *Francisella philomiragia* (Copeland et al., 2008). Like *F. tularensis* subsp. *novicida*, *F. philomiragia* is a water-associated bacterium and less pathogenic to humans than *F. tularensis* subsp. *tularensis* and *holarctica* (Penn, 2005). Our infection experiments indicate that the *capBCA* locus is necessary for *the in vivo* fitness and full virulence of *F. tularensis* types A and B organisms. Weiss et al. (2007) also showed that the transposon mutants in the *capB* and *capC* genes of *F. tularensis* subsp. *novicida* are attenuated in a systemic infection mouse model. Considering that mammals may not be the natural reservoirs of *F. tularensis* (Oyston and Quarry, 2005), the uncharacterized function(s) of the *capBCA* locus in *F. tularensis* and perhaps other *Francisella* species may be evolutionarily selected for a survival advantage under non-mammalian conditions.

It is noticeable that the attenuation level of the Schu S4 Δ*capBCA* mutant was significantly lower than that of the LVS counterpart in our intranasal infection model in BALB/c mice. This difference may be due to other Schu S4-specific factor(s) that might have overshadowed the impact of the *capBCA* locus in the mouse model. Laboratory mice are exceptionally susceptible to Schu S4 infection (Rick Lyons and Wu, 2007). Alternatively, the *capBCA* locus operates in a different manner at the transcriptional and/or post-transcriptional level. Lindgren et al. (2007) recently reported that a deletion in the catalase-encoding *katG* of *F. tularensis* LVS led to significant attenuation in virulence, but a similar Schu S4 Δ*katG* mutant showed no attenuation in virulence. Finally, different mouse strains and routes of infection have yielded variable outcomes with the *F. tularensis capB* mutants in terms of virulence

and immuno-protection. Michell et al. (2010) reported more than 100-fold attenuation with an Schu S4 *capB* mutant following subcutaneous inoculation of BALB/c mice, whereas no significant attenuation was observed with an independent Schu S4 *capB* mutant when BALB/c mice were infected by aerosol inoculation (Conlan et al., 2010). Further evaluation of the *capBCA* mutants in other animal models is warranted to conclusively define the contribution of this gene locus to *F. tularensis* infection in humans.

ACKNOWLEDGMENTS

This work was supported by the Tsinghua University Support Fund (Jing-Ren Zhang), the Chinese Universities Scientific Fund No. 2009-3-03 (Jingliang Su), Commonwealth of Kentucky Research Challenge Trust Fund (Yousef Abu-Kwaik), and NIH grants R21AI083963 (Jing-Ren Zhang), P01AI056320 (Jing-Ren Zhang), R01AI43965 (Yousef Abu-Kwaik), and R01AI069321 (Yousef Abu-Kwaik).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 09 December 2010; accepted: 07 April 2011; published online: 25 April 2011.

Citation: Su J, Asare R, Yang J, Nair MKM, Mazurkiewicz JE, Abu-Kwaik Y and Zhang J-R (2011) The capBCA locus is required for intracellular growth of Francisella tularensis LVS. Front. Microbio. ***2****:83. doi: 10.3389/fmicb.2011.00083*

This article was submitted to Frontiers in Cellular and Infection Microbiology, a specialty of Frontiers in Microbiology.

APPENDIX

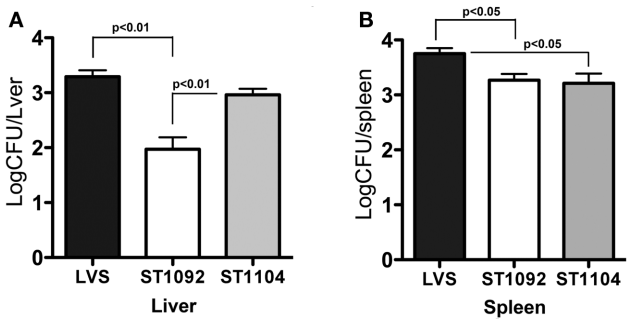

FIGURE A1 | *In vivo* growth of the *F. tularensis* Δ*capB* mutants in the livers (A) and spleens (B) of BALB/c mice. BALB/c mice were infected individually with LVS (4,500 CFUs), ST1092 (5,100 CFUs), or ST1104 (4,800 CFUs) by intranasal inoculation. Bacterial levels in the livers and spleens were assessed at day 7 as in **Figure 3**.