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Contribution of Citrulline Ureidase to *Francisella tularensis* Strain Schu S4 Pathogenesis[▽]

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The citrulline ureidase (CTU) activity has been shown to be associated with highly virulent *Francisella tularensis* strains, including Schu S4, while it is absent in avirulent or less virulent strains. A definitive role of the *ctu* gene in virulence and pathogenesis of *F. tularensis* Schu S4 has not been assessed; thus, an understanding of the significance of this phenotype is long overdue. CTU is a carbon-nitrogen hydrolase encoded by the citrulline ureidase (*ctu*) gene (FTT0435) on the *F. tularensis* Schu S4 genome. In the present study, we evaluated the contribution of the *ctu* gene in the virulence of category A agent *F. tularensis* Schu S4 by generating a nonpolar deletion mutant, the Δ*ctu* mutant. The deletion of the *ctu* gene resulted in loss of CTU activity, which was restored by transcomplementing the *ctu* gene. The Δ*ctu* mutant did not exhibit any growth defect under acellular growth conditions; however, it was impaired for intramacrophage growth in resting as well as gamma interferon-stimulated macrophages. The Δ*ctu* mutant was further tested for its virulence attributes in a mouse model of respiratory tularemia. Mice infected intranasally with the Δ*ctu* mutant showed significantly reduced bacterial burden in the lungs, liver, and spleen compared to wild-type (WT) Schu S4-infected mice. The reduced bacterial burden in mice infected with the Δ*ctu* mutant was also associated with significantly lower histopathological scores in the lungs. Mice infected with the Δ*ctu* mutant succumbed to infection, but they survived longer and showed significantly extended median time to death compared to that shown by WT Schu S4-infected mice. To conclude, this study demonstrates that *ctu* contributes to intracellular survival, in vivo growth, and pathogenesis. However, *ctu* is not an absolute requirement for the virulence of *F. tularensis* Schu S4 in mice.

Francisella tularensis, the etiological agent of tularemia, is a category A bioterrorism agent. High infectivity, ease of intentional aerosol dissemination, and lack of a licensed vaccine have made *Francisella* a potential biowarfare agent (5, 12, 34). The two major subspecies of *Francisella* have been divided on the basis of virulence, epidemiological distribution, and biochemical reactions (51). *F. tularensis* subspecies *tularensis* (type A strain) is highly virulent and the major cause of tularemia in North America, whereas *F. tularensis* subspecies *holarctica* (type B strain), prevalent in Europe and Asia, is less virulent. Biochemically, type A strains produce acid from glycerol and exhibit citrulline ureidase (CTU) activity, while type B strains do not exhibit these activities (21). In contrast to these biochemical differences, very limited variation is seen at the genetic level (25, 41), suggesting that differences in virulence between type A and B strains may arise from differential gene expression by nearly homologous genomes. The highly virulent Schu S4 strain represents type A *F. tularensis* subspecies *tularensis* and was originally isolated from a clinical case of tularemia in Ohio in 1941. To date, only a few virulence-associated genes have been characterized in this strain (22, 36, 37, 48), and its virulence determinants still remain poorly understood.

CTU, a member of the carbon-nitrogen hydrolase family protein encoded by the *F. tularensis* genome (FTT0435), degrades citrulline into ornithine, carbon dioxide, and ammonia (10). Citrulline is generated during the catabolism of arginine by bacterial arginine deiminase (ADI) (40, 47). Ornithine generated by citrulline degradation is either exchanged for arginine by an arginine-ornithine transporter or utilized for the generation of polyamines and energy in the form of ATP (40). Citrulline is also produced by macrophages during conversion of L-arginine and oxygen to nitric oxide (NO) by inducible NO synthase (iNOS). Citrulline thus formed can be recycled to L-arginine through an arginine-citrulline cycle, which not only regulates intracellular availability of L-arginine but, in turn, maintains a sustained production of NO by macrophages (19). However, unlike citrulline, macrophages have little or no capacity to convert ornithine, the breakdown product of citrulline into L-arginine (4). Recent reports have demonstrated that reactive nitrogen species derived from NO are critical for clearance of *F. tularensis* (27, 29). In addition, ammonia generated by degradation of citrulline has been proposed to play a role in alkalization of endosomal pH leading to phagosomal maturation arrest (25). Thus, interruption of the arginine-citrulline cycle through the degradation of citrulline into ornithine, CO_2, and ammonia by CTU may assume an important role in the virulence of *F. tularensis.*

Until recently, CTU activity has been used to differentiate strains of *F. tularensis* with high virulence from strains with low virulence or avirulent strains (45). Previous studies have shown that the majority of virulent *F. tularensis* type A strains exhibit high CTU activity while strains lacking this enzyme activity are either less virulent or avirulent (10, 11). However, a direct relationship between CTU activity and virulence of *F. tularensis* could not be established. A majority of these previous studies were based on comparisons of CTU activity in naturally

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TABLE 1. Primer sequences, bacterial strains, and plasmids used and generated in this study

Primer, strain, or plasmid	Primer sequence	Source or reference
Primers		
Up ctuF (A)	5′ CACGCGTCGACACATTCAAATGGTCAAGGAGTTTT 3′	
Up ctuR (B)	5′ ATCAAATCTCCTTTATAGCCGGT 3′	
Dn ctuF (C)	5′ CGGCTATAAAGGAGATTTGATATTGATAAAAATGTTTTTAGTTTAGAG 3′	
Dn ctuR (D)	5′ GGGACTAGTTCATCTAAACGCTAATCATGCTG 3′	
CTUpKKF	5′ AAAACTGCAGATGGCGAATATAAAAGTTGCAG 3′	
CTUpKKR	5′ TTAATACTTTCTAACAATTTCTTCA 3′	
pKK kan CTU-F	5′ TGAAGAAATTGTTAGAAAGTATTAAGTCGACTATTAAAAAAATTCAT CAAG 3′	
pKK kan CTU-R PstI	5′ GGAGTAACTGCAGTATGTCACAT 3′	
Strains or plasmids[a]		
F. tularensis Schu S4		USAMRIID
pDMK		27
pDMK::Δ*ctu*		This study
S17-1::pDMK::Δ*ctu*		This study
F. tularensis Schu S4::Δ*ctu* (Δ*ctu*)		This study
F. tularensis Schu S4::Δ*ctu*+p*ctu*		This study

[a] *E. coli* S17-1 available in the Center for Immunology and Microbial Disease, Albany Medical College, was used for conjugation experiments.

occurring wild-type (WT) virulent type A strains with that in less virulent or avirulent type B variants of *F. tularensis*. In the current study, a genetic approach was used to directly assess the role of CTU activity in the pathogenesis and virulence of the *F. tularensis* Schu S4 strain.

MATERIALS AND METHODS

Bacterial strains. *F. tularensis* Schu S4, originally isolated from a human case of tularemia, was obtained from the U.S. Army Medical Research Institute for Infectious Diseases (USAMRIID; Frederick, MD). *F. tularensis* live vaccine strain (LVS; ATCC 29684; American Type Culture Collection, Rockville, MD) was kindly provided by Karen Elkins (U.S. Food and Drug Administration, Bethesda, MD). The bacteria were cultured on modified Mueller-Hinton (MH) chocolate agar plates (2, 13) or in MH broth (Difco Laboratories, Lawrence, KS) supplemented with ferric pyrophosphate and Iso-Vitalex (BD Biosciences, San Jose, CA). Active mid-log-phase bacteria were harvested and stored in liquid nitrogen; 1-ml aliquots were thawed periodically for use.

Generation of the Δ*ctu* mutant and transcomplementation. *F. tularensis* Schu S4 was used for the generation of an in-frame gene deletion mutant of the *ctu* gene (Δ*ctu*). All genetic manipulations of the Schu S4 strain conformed to Centers for Disease Control guidelines and were performed in a biosafety level 3/animal biosafety level 3 facility at Albany Medical College. The sequences and locations of the primers, bacterial strains used, and plasmid constructs generated in this study are shown in Table 1. An allelic replacement method was adapted for the generation of the Δ*ctu* mutant of *F. tularensis* Schu S4 (15). A suicide plasmid vector, pDMK, kindly provided by Anders Sjostedt (University of Umeå, Sweden) was used for mutagenesis (27). A previously described splicing by overlap extension PCR method was used to generate a deletion within the coding region of the *ctu* gene in such a way that only the flanking regions of the gene remained (26). The PCR-amplified fragment containing up- and downstream regions minus the coding region of the *ctu* gene was cleaved with SalI/SpeI restriction enzymes and ligated into a similarly digested pDMK vector. The resultant pDMK::Δ*ctu* was transformed into chemically competent *Escherichia coli* S17-1 cells to yield *E. coli* pDMK::Δ*ctu*, and the colonies were selected on Luria-Bertani (LB) plates containing kanamycin (20 μg/ml). Early-log-phase cultures of *E. coli* pDMK::Δ*ctu* (~10^7 CFU/ml) and *F. tularensis* Schu S4 (~10^9 CFU/ml) grown in Chamberlain's chemically defined medium (7) were prepared for conjugation according to the method described earlier (2, 13). The transconjugants were selected on modified chocolate agar plates containing kanamycin (10 μg/ml) and polymyxin B (100 μg/ml) (2). The resultant colonies were screened for loss of both resistance to kanamycin and sensitivity to sucrose. The mutant colonies exhibiting such a phenotype were selected, and deletion of *ctu* was confirmed by PCR by using flanking primers and DNA sequencing. The Δ*ctu* mutant was further characterized for its virulence attributes by macrophage invasion assays and mouse survival studies.

For transcomplementation of the Δ*ctu* mutant, a pKK214::*gfp* vector expressing green fluorescent protein kindly provided by T. Kawula (University of North Carolina, Chapel Hill, NC) was used. The *ctu* gene was cloned downstream of the *F. tularensis* GroEL promoter by replacing *gfp* in the pKK214::*gfp* vector. Briefly, the *ctu* gene was amplified using Schu S4 genomic DNA as a template employing primers CTUpkkF and CTUpkkR (Table 1). Simultaneously, a kanamycin cassette was amplified from plasmid pkk214::*gfp* using the pkk kan CTU-F and pkk kan CTU-R PstI primers (Table 1). Both the *ctu* gene and kanamycin cassette PCR products were fused together by overlap extension. The final PCR product was digested with PstI and ligated into the similarly digested pKK214. This method allowed us to replace *gfp* with the *ctu* gene while keeping the kanamycin gene in frame. This construct was termed p*ctu* and checked for the orientation of the *ctu* gene by PCR. The p*ctu* containing the cloned *ctu* gene in the correct orientation was electroporated into the Δ*ctu* mutant as described earlier (3) to generate the transcomplemented Δ*ctu*+p*ctu* strain. The expression of CTU in Δ*ctu*+p*ctu* was confirmed by reverse transcriptase PCR (RT-PCR) and a citrulline ureidase activity assay.

For RT-PCR, RNA was isolated from overnight bacterial cultures by using the Trizol reagent (Invitrogen, Carlsbad, CA), and 1 μg of RNA was reverse transcribed using *ctu* gene-specific primers (CTUpkkR) by SuperScript II RT kit (Invitrogen, Carlsbad, CA). The cDNA was amplified using the CTUpkkF and CTUpkkR primers. The amplified products were electrophoresed on a 1.5% agarose gel and visualized on a UV transilluminator after staining with ethidium bromide.

CTU activity assay. For assessment of CTU activity in the WT *F. tularensis* Schu S4, Δ*ctu*, and Δ*ctu*+p*ctu* strains, a thin-layer chromatography (TLC)-based approach as described earlier was used (20, 24). The bacterial cultures were resuspended in 25 μl of 0.1 M phosphate-buffered saline (PBS; pH 6.5) to yield a concentration of 1×10^{10} CFU/ml and lysed by ultrasonication. The lysates were filtered using a 0.22-μm filter. Forty microliters of the filtrate was incubated with an equal volume of 0.7% (wt/vol) citrulline (Sigma, St. Louis, MO) at 30°C for 20 h. Three microliters of the reaction mix was spotted onto silica gel TLC plates (Partisil Diamond K6F; Schleicher & Schuell, Keene, NH). The spots were dried, and the TLC was carried out using *n*-butanol–acetic acid–water solvents mixed at a ratio of 40:10:17 in a glass chamber until the liquid front reached the top of the plates. The plates were removed from the glass chamber, dried, and sprayed with 0.5% ninhydrin dissolved in *n*-butanol. The plates were dried again in a fume hood and developed at 60°C for 30 min to visualize the colored spots. *F. tularensis* LVS, which does not exhibit CTU activity (38, 39), and bacterial lysates not treated with citrulline were used as negative controls. Citrulline (0.7% [wt/vol]) and ornithine (100 mM) were spotted as positive controls.

Growth curves. WT Schu S4 and the Δ*ctu* and the Δ*ctu*+p*ctu* strains were cultured in MH broth for 48 h at 37°C in a shaking incubator. Aliquots were

withdrawn at 4-h intervals, and absorbance was recorded at 600 nm. To enumerate CFU, the aliquots were serially diluted in sterile PBS and plated onto MH chocolate agar plates. The plates were incubated for 48 h, and the colonies were counted and expressed as $\log_{10}$ CFU/ml.

Macrophage invasion assay. To address the effect of *ctu* gene deletion on intramacrophage survival, a macrophage cell culture invasion assay was performed as described earlier (23, 31, 32). Bone marrow-derived macrophages (BMDMs) isolated from WT and *inos*$^{-/-}$ C57BL/6 mice and the MH-S cell line, a murine alveolar macrophage cell line (33), were used in these assays. MH-S cells or BMDMs were either left untreated or treated with recombinant gamma interferon (IFN-γ; 100 ng/ml; Sigma, St. Louis, MO) for 16 h prior to infection, and thereafter. The macrophages were infected with WT Schu S4 or the Δ*ctu* or Δ*ctu*+p*ctu* strain at a multiplicity of infection of 100. The infection was synchronized by centrifuging the plates at 1,000 × *g* for 5 min at 4°C. Two hours after infection, the growth medium was replaced with medium containing gentamicin (100 μg/ml) to kill all adherent and extracellular bacteria. One hour later, the medium containing gentamicin was replaced with growth medium without any antibiotics, and the cells were incubated at 37°C in the presence of 5% CO_2. The cells were lysed with 0.1% sodium deoxycholate 24 and 48 h later, diluted 10-fold in sterile PBS and spread onto chocolate agar plates (BD Biosciences, San Jose, CA) to quantitate the number of bacteria that replicated intracellularly. The Δ*ctu*+p*ctu* strain was plated onto chocolate agar plates containing kanamycin (10 μg/ml) to ensure that recovered bacteria still carried the p*ctu* plasmid. The results were expressed as $\log_{10}$ CFU/ml.

Measurement of NO. The concentration of nitrite (NO_2^-), the oxidized metabolite of NO, was assessed by the Griess reaction. The culture supernatants of BMDMs unstimulated or stimulated with 100 ng/ml of IFN-γ and infected with the Δ*ctu* mutant or WT Schu S4 were collected at 24 and 48 h and analyzed for NO_2^- levels. One hundred microliters of each culture supernatant was mixed with an equal volume of Griess reagent (Promega, Madison, WI) and incubated at room temperature for 10 min in the dark. The optical density readings were recorded at 545 nm. A standard curve generated with various concentrations (2.5 to 10 μM) of sodium nitrite ($NaNO_2$) was used for determining NO_2^- concentrations in culture supernatants. The data were expressed as μM concentrations of NO_2^-.

Mice experiments. All experiments were conducted using 6- to 8-week-old BALB/c mice (Taconic, Germantown, NY) of both sexes. The mice were maintained in a specific-pathogen-free environment in the Animal Resource Facility at Albany Medical College. All Schu S4 challenge experiments were performed in a CDC-approved animal biosafety level 3 facility at Albany Medical College and conformed to the Institutional Animal Care and Use Committee guidelines.

Kinetics of bacterial clearance. The effect of the *ctu* mutation on bacterial survival under in vivo conditions was determined by performing a kinetic experiment in mice. Six- to eight-week-old BALB/c mice were infected intranasally with 25 CFU of WT Schu S4 or the Δ*ctu* or Δ*ctu*+p*ctu* strain. Mice were sacrificed on day 1, 3, or 5 postinfection (PI), and bacterial numbers were quantified in the lung, liver, and spleen of the infected mice. Briefly, the organs were subjected to mechanical homogenization using a Mini-BeadBeater-8 (BioSpec Products Inc. Bartlesville, OK). The tissue homogenates were spun at 1,000 × *g* for 10 s in a microcentrifuge to pellet tissue debris. The supernatants were diluted 10-fold in sterile PBS, and 10 μl of each dilution was spotted onto MH chocolate agar plates in duplicate and incubated at 37°C for 48 to 72 h in the presence of 5% CO_2. The colonies on the plates were counted and expressed as CFU per organ as reported earlier (1, 49).

Histopathology. The lungs from WT Schu S4-, Δ*ctu* strain-, or Δ*ctu*+p*ctu* strain-infected BALB/c mice were excised and fixed in 10% neutral buffered formalin for histological evaluation. The lungs were collected on days 1, 3, and 5 PI. The lungs were inflated via instillation of PBS into the trachea prior to fixation and processed using standard histological procedures. The paraffin-embedded sections were stained with hematoxylin-eosin and examined by light microscopy. The hematoxylin-eosin-stained sections were analyzed in a blind fashion using a histopathological scoring system described earlier (1).

Survival experiments. To analyze the role of *ctu* in virulence, time-to-death experiments were performed. BALB/c mice were deeply anesthetized via intraperitoneal injection of a cocktail of ketamine (Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (Phoenix Scientific, St. Joseph, MO). Mice were challenged intranasally with 25 CFU of WT Schu S4 or the Δ*ctu* or Δ*ctu*+p*ctu* strain in a volume of 20 μl PBS (10 μl/nare). The mice were monitored for a period of 21 days for morbidity and mortality. The survival results were plotted as Kaplan-Meier curves, and the statistical significance was determined by log-rank test.

Statistical analysis. All results were expressed as means ± standard errors of the means, and comparisons between the groups were made using one-way analysis of variance (ANOVA) followed by Bonferroni's correction, the non-parametric Mann-Whitney test, or Student's *t* test. The survival data were analyzed using log-rank test, and *P* values were determined. Differences between the experimental groups were considered significant at a *P* value of <0.05.

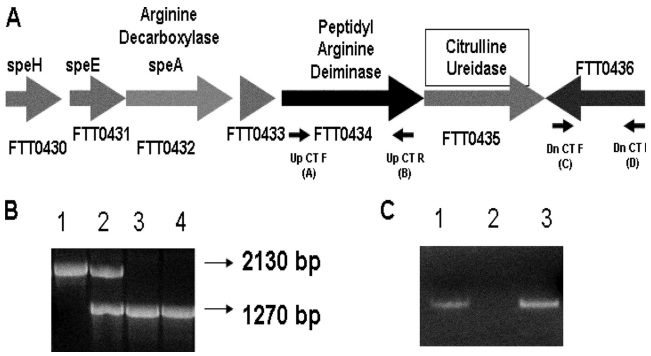


FIG. 1. Verification of *ctu* gene deletion in *F. tularensis* Schu S4. (A) Genomic organization of the *ctu* gene (FTT0435) of Schu S4. Small arrows indicate primer locations. (B) Confirmation of the *ctu* gene deletion by PCR. Flanking primers A plus D were used for confirmation of the *ctu* gene deletion. Lane 1, WT *F. tularensis* Schu S4; lane 2, a merodiploid stage indicating integration of pDMK::Δ*ctu* in the Schu S4 genome; lanes 3 and 4, the Δ*ctu* mutant. An amplification product of ~1.27 kbp (lanes 3 and 4) compared to 2.13 kbp in WT Schu S4 (lane 1) confirmed the *ctu* gene deletion. (C) RT-PCR analysis. Lane 1, WT Schu S4; lane 2, the Δ*ctu* mutant; lane 3, the Δ*ctu*+p*ctu* transcomplemented strain.

RESULTS

The *ctu* gene is interrupted in avirulent or less virulent strains of *F. tularensis*. The CTU protein in strain Schu S4 is 286 amino acids long and has a molecular mass of 32.29 kDa. Computer-based comparative analysis showed that the amino acid sequences of CTU from Schu S4 and those from virulent type A strains FSC198 and WY96-3418 were 100% identical. The CTU sequence of type A strains also exhibited 97% sequence homology with the attenuated *F. tularensis* subspecies *holarctica* type B LVS, FTA, and OSU18. However, unlike the single open reading frame (ORF) of type A strains, the CTU ORF in type B strains, including LVS, was found to be interrupted by stop codons at amino acids 54 and 141, resulting in a truncated protein product. In addition, the CTU sequence in LVS and other type B strains revealed amino acid changes/substitutions at positions 113, 136, 183, 204, 222, and 248. Thus, sequence analysis revealed that while the virulent type A strains of *F. tularensis* are all strongly CTU positive owing to the presence of an uninterrupted ORF, the avirulent or less virulent type B strains are CTU negative due to mutations in the *ctu* gene. The findings indicate that *ctu* gene sequence analysis may form a strong basis for the rapid differentiation of type A and B strains of *F. tularensis*.

Verification of *ctu* gene deletion in *F. tularensis* Schu S4. The genomic organization of the *ctu* gene is shown in Fig. 1A. Deletion of the *ctu* gene in the Δ*ctu* mutant was confirmed by PCR, DNA sequencing, and RT-PCR. A colony PCR using primer pairs located up- and downstream of the *ctu* gene resulted in a smaller fragment (~1.27 kb) in the Δ*ctu* mutant than in the WT Schu S4 (~2.13 kb), confirming the gene deletion (Fig. 1B). DNA sequencing of the regions flanking the deleted *ctu* gene revealed that deletion of the *ctu* gene was in

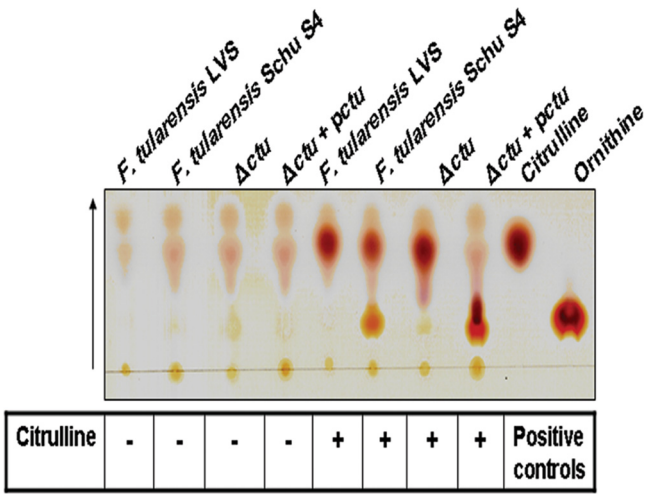


FIG. 2. CTU activity assay. The CTU activity assay was performed as described in Materials and Methods. Citrulline and ornithine were run as positive controls. The arrow indicates the direction of run.

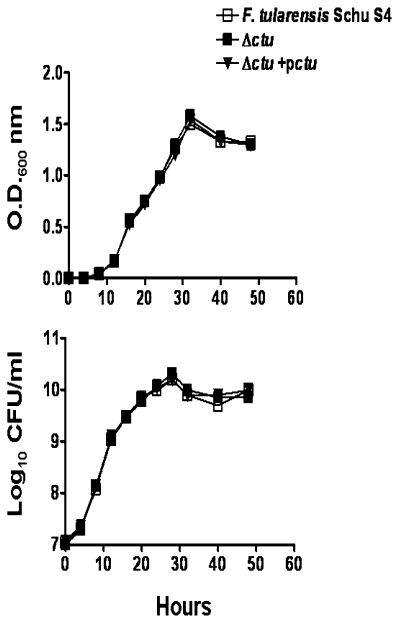


FIG. 3. In vitro growth analysis of the Δ*ctu* mutant of *F. tularensis* Schu S4. The growth curve for the Δ*ctu* mutant was generated and compared with those of WT Schu S4 and the Δ*ctu*+p*ctu* transcomplemented bacteria. The optical density at 600 nm ($O.D._{600}$nm) (upper panel) and the corresponding CFU (lower panel) were recorded at the indicated times.

frame and ORFs up- and downstream of the *ctu* gene were unaltered (data not shown). RT-PCR was performed using *ctu* gene-specific primers. This analysis also confirmed deletion of the *ctu* gene, as no transcripts were amplified in the Δ*ctu* mutant; however, *ctu*-specific transcripts were observed in WT Schu S4 and the transcomplemented strain (Fig. 1C).

Deletion of the *ctu* gene results in loss of CTU activity. We further characterized the Δ*ctu* mutant for CTU activity by TLC. The lysates from WT Schu S4 degraded citrulline into ornithine, whereas the Δ*ctu* mutant, similar to *F. tularensis* LVS, lost its citrulline degrading capability. CTU activity was restored by complementing the *ctu* gene in *trans* in the Δ*ctu*+p*ctu* strain (Fig. 2). The results demonstrate that the *ctu* gene in Schu S4 is required for degradation of citrulline into ornithine and that this function is specific to the *ctu* gene. The results also confirm findings from sequence analysis that LVS does not have a functional *ctu* gene.

Loss of CTU does not affect acellular growth. The role of the *ctu* gene under acellular growth conditions was assessed by comparing the growth curve of the Δ*ctu* mutant with those of WT Schu S4 and the Δ*ctu*+p*ctu* transcomplemented strain. The Δ*ctu* mutant did not exhibit any growth defect, and its growth rate was similar to those of WT Schu S4 and the transcomplemented strain (Fig. 3). This result suggests that CTU activity is not required for growth under normal, acellular growth conditions.

Loss of *ctu* attenuates intramacrophage survival. On the basis of the association of CTU activity with highly virulent type A strains of *F. tularensis*, we hypothesized that deletion of the *ctu* gene would lead to attenuation of intramacrophage growth. We performed macrophage cell culture invasion assay in BMDMs and the MH-S cell line, using WT Schu S4, the Δ*ctu* mutant, and the Δ*ctu*+p*ctu* transcomplemented strain for the quantitation of intramacrophage survival and replication. Despite equal numbers of bacteria recovered at 3 h PI, significantly lower numbers (five- to sevenfold) of Δ*ctu* mutants, relative to the number of WT Schu S4 and the transcomplemented strain, were recovered in BMDMs and the MH-S cells at 24 and 48 h PI (Fig. 4, left panels). Significantly reduced numbers of the Δ*ctu* mutant compared to the WT and transcomplemented strain were also recovered from IFN-γ-treated BMDMs and MH-S cells (Fig. 4, right panels). Transcomplementation restored growth of the Δ*ctu* mutant within BMDMs and MH-S cells to levels intermediate between the Δ*ctu* mutant and the WT *F. tularensis* Schu S4 strain. These results demonstrate that CTU contributes to the intramacrophage survival of *F. tularensis* Schu S4. However, in the absence of a complete clearance of the Δ*ctu* mutant by infected macrophages, our results indicate that *ctu* is not the sole factor responsible for the intracellular lifestyle of *F. tularensis* Schu S4 and that other factors in conjunction with *ctu* contribute to its intramacrophage survival and replication.

Enhanced killing of the Δ*ctu* mutant may be attributed to NO production in infected macrophages. We hypothesized that suppression of NO production by CTU via interruption of the arginine-citrulline cycle would enhance intramacrophage survival of *F. tularensis* Schu S4. Since reactive nitrogen species are instrumental in the intramacrophage killing of *F. tularensis* (27–29), to examine whether the enhanced killing of the Δ*ctu* mutant relative to that of the WT Schu S4 strain was due to differences in the levels of NO produced by infected macrophages, levels of nitrite/nitrate, the stable oxidative product of NO, were measured by the Griess reaction and used as an indicator of NO production in culture supernatants of infected macrophages with or without IFN-γ treatment. Elevated nitrite levels were observed in culture supernatants of the Δ*ctu* strain-infected BMDMs compared to those observed in WT Schu S4-infected cells (Fig. 5A). Conversely, in BMDMs deficient for iNOS, intramacrophage survival of the Δ*ctu* mutant

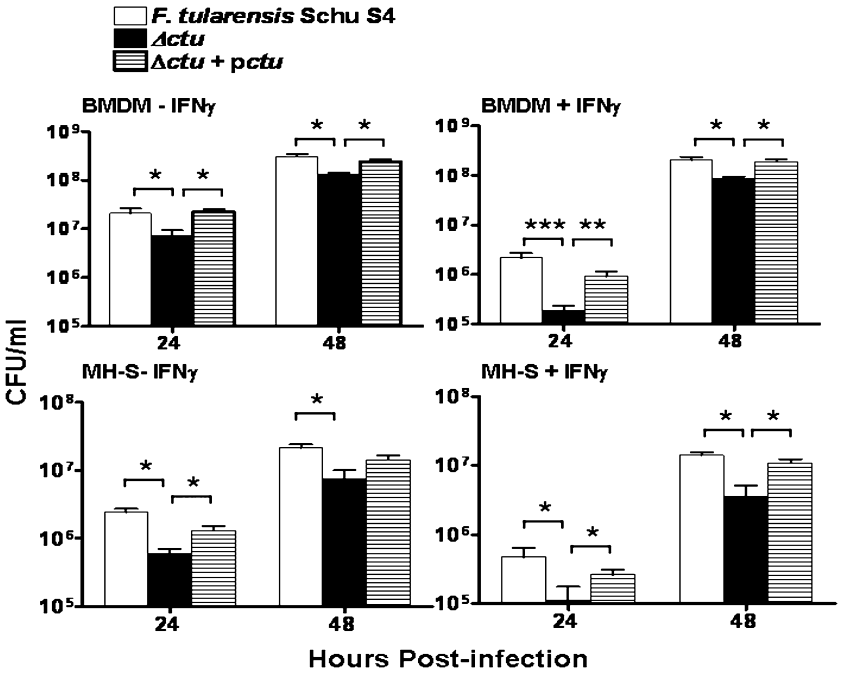


FIG. 4. The Δ*ctu* mutant of Schu S4 is deficient for intramacrophage survival. A macrophage cell culture invasion assay was performed on BMDMs and the MH-S cells untreated (left panels) or treated with IFN-γ (right panels). The intracellular replication was quantitated at the indicated times and expressed as CFU/ml. The values represent the means ± standard errors (SE) of quadruplicate samples and are cumulative of the results of three experiments conducted. *P* values were determined using one-way ANOVA. ⋆, $P < 0.05$; ⋆⋆, $P < 0.01$; and ⋆⋆⋆, $P < 0.001$.

was restored, similar to that of WT Schu S4 (Fig. 5B). These results demonstrate that NO contributes to the enhanced intramacrophage killing of the Δ*ctu* mutant.

Δ*ctu* mutant-infected mice exhibit significantly reduced bacterial burden and histopathology. It was next investigated whether the enhanced killing of the Δ*ctu* mutant in macrophages could be replicated under in vivo conditions. BALB/c mice were infected intranasally with 25 CFU of WT Schu S4, Δ*ctu* mutant, or Δ*ctu*+p*ctu* transcomplemented bacteria. Mice were sacrificed at the indicated times above, and bacterial burdens were quantitated in the lung, liver and spleen. At days 3 and 5 PI, at which 100% of Schu S4-infected mice succumbed to infection, significantly lower bacterial loads were observed in the lung, liver, and spleen of the Δ*ctu* mutant-infected mice than in the Schu S4-infected counterparts (Fig. 6A). The transcomplemented strain, the Δ*ctu*+p*ctu* strain, exhibited partial restoration of the parental Schu S4 phenotype and was recovered in nearly 10-fold-higher numbers in the lungs at day 3 PI, and in the liver and spleen at day 5 PI, than was the Δ*ctu* mutant strain. These results demonstrate that *ctu*, in addition to its role in intramacrophage survival, is required for in vivo replication of *F. tularensis* Schu S4.

Histological lesions in the lungs of mice infected with the Δ*ctu* mutant were quantitated using a previously described histopathological scoring (HPS) system (1, 31), and the scores were compared with those in the WT Schu S4- and Δ*ctu*+p*ctu* strain-infected mice. Consistent with the reduced bacterial burdens, histopathological scores observed in the lungs at days 3 and 5 PI (Fig. 6B), and in the liver and spleen at day 3 PI, of mice infected with the Δ*ctu* mutant were significantly lower than those observed in Schu S4-infected mice (data not shown). Transcomplementation increased the severity of the lung lesions in the Δ*ctu*+p*ctu* strain-infected mice compared to that in the Δ*ctu* mutant-infected mice. However, the severity of the lesions in the Δ*ctu*+p*ctu* strain-infected mice never reached the extent observed in the WT Schu S4-infected mice. Collectively, these results demonstrate that *ctu* participates in the pathogenesis of *F. tularensis* Schu S4 strain.

The Δ*ctu* mutant of Schu S4 is partially attenuated for virulence in mice. An attenuation in intramacrophage survival and reduced bacterial burden in mice infected with the Δ*ctu* mutant prompted us to further investigate the effect of *ctu* gene deletion on virulence in mice, using an intranasal challenge protocol (1, 2, 31, 32). BALB/c mice are extremely susceptible to Schu S4 infection, and a dose as low as 1 CFU administered intranasally can cause death in the infected mice (22; our unpublished data). Groups of 15 mice were each inoculated intranasally with 25 CFU of WT Schu S4, the Δ*ctu* strain, or the transcomplemented strain. The mice were monitored twice daily for morbidity and mortality for a period of 21 days. All mice inoculated with 25 CFU of Schu S4 succumbed to infection by day 5 PI. Although 100% of the mice infected with 25 CFU of the Δ*ctu* strain also succumbed to infection, a significantly extended median time to death compared to that of Schu S4-infected mice was observed (Fig. 7). Mice infected with the Δ*ctu*+p*ctu* transcomplemented strain had an intermediate virulence phenotype. The results suggest that the Δ*ctu* strain undergoes slow replication in infected mice compared to WT Schu S4 strain; however, given the extremely high virulence and very low 100% lethal dose of Schu S4, subsequent increases in bacterial numbers are sufficient to cause death in

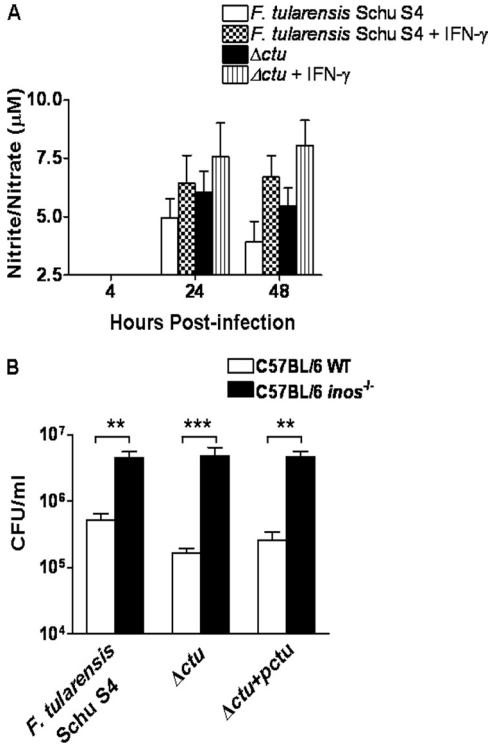


FIG. 5. Enhanced intramacrophage killing of the Δ*ctu* mutant is NO dependent. (A) Culture supernatants from the invasion assay were analyzed for nitrite levels, using a commercial Griess reagent. The detection limit of the Griess reaction was 2.5 μM. The results are expressed as the means ± SEs from four replicate samples tested in triplicate and are representative of the results of two independent experiments conducted. (B) BMDMs derived from C57BL/6 WT and *inos*$^{-/-}$ mice were stimulated with IFN-γ (100 ng/ml) for 16 h prior to and after infection with the *F. tularensis* Schu S4, Δ*ctu*, or Δ*ctu*+p*ctu* strain at a multiplicity of infection of 100. The infected BMDMs were lysed 24 h PI, and the bacterial numbers were quantitated. The results are expressed as the means ± standard deviations. The statistical analysis was performed using unpaired Student's *t* test. **, $P < 0.01$; and ***, $P < 0.001$.

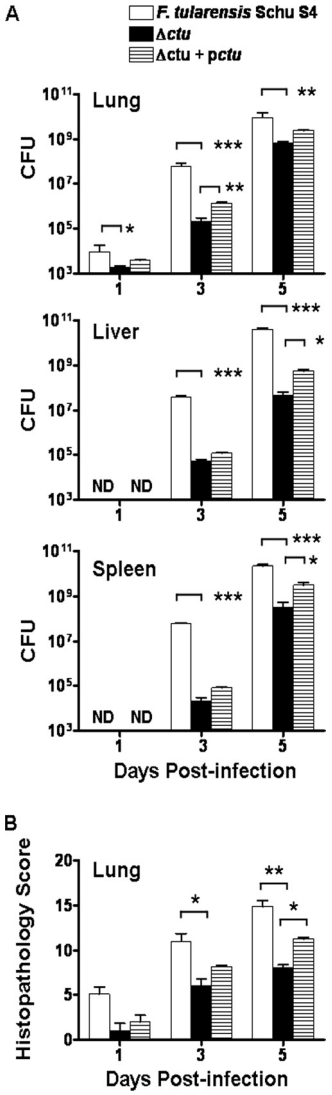


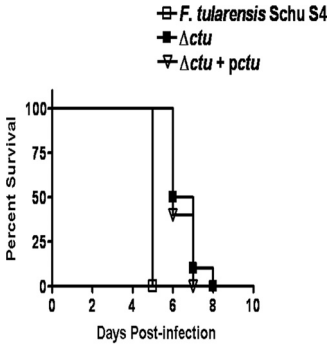
FIG. 6. Quantitation of bacterial burden and histopathological lesions. Mice ($n = 4$ to 6 per group), infected with 25 CFU of WT Schu S4, the Δ*ctu* mutant, and the transcomplemented Δ*ctu*+p*ctu* strain were sacrificed at the indicated times. The bacterial burden in the lungs, liver, and spleen (A) and the histopathological lesions in the lungs (B) were quantitated. The results are expressed as the means ± SEs and are cumulative of the results of two independent experiments conducted. *P* values were determined using one-way ANOVA. ND, not detected; *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

the infected mice. These results also indicate that the *ctu* gene deletion causes only a partial attenuation of virulence in mice.

DISCUSSION

CTU activity has essentially been used as a marker to differentiate highly virulent strains of *F. tularensis* from less virulent or avirulent strains (10, 11, 38, 39, 42). Despite this exclusive association with a highly virulent phenotype, the actual contribution of CTU to virulence and pathogenesis of *F. tularensis* is not known. We attempted to address this important issue by generating a nonpolar *ctu* deletion mutant of the highly virulent Schu S4 strain of *F. tularensis* and further characterized this mutant for its virulence attributes in macrophages and mice. DNA sequence analysis of the Δ*ctu* mutant revealed that deletion of the *ctu* gene was in frame and did not alter the transcription of upstream genes (data not shown), as *ctu* is the last gene of the operon. Our in vitro analysis confirmed that the Δ*ctu* mutant was not growth defective under acellular conditions. However, the Δ*ctu* mutant was attenuated for intramacrophage survival and showed reduced virulence in intranasally infected mice.

Francisella utilizes L-arginine as a carbon and/or nitrogen source (7). The *ctu* gene of *F. tularensis* Schu S4 is carried on an operon that resembles the ADI system required for arginine utilization in several bacterial pathogens (6, 9, 18). The *ctu* (FTT0435), arginine deiminase (FTT0434), and arginine decarboxylase (*speA* [FTT0432]) genes similar to those found on other bacterial ADI operons may serve to carry out arginine catabolism in *Francisella*, whereas spermidine synthase (*speE* [FTT0431]) and *S*-adenosylmethionine decarboxylase (*speH* [FTT0430]) genes are required for polyamine biosynthesis (Fig. 1A). A recent report has shown that transcription of all



Bacterial Strain	Median Survival time (Days)
F. tularensis Schu S4	5
Δ*ctu*	6.5*
Δ*ctu* + p*ctu*	6

* Significantly different from *F. tularensis* Schu S4 (P<0.006)

FIG. 7. Mouse survival studies. Six- to eight-week old BALB/c mice ($n = 15$ per group) were infected intranasally with 25 CFU of WT Schu S4, the Δ*ctu* mutant, and the Δ*ctu*+p*ctu* transcomplemented strain. The mice were monitored for morbidity and mortality for a period of 21 days. The results are expressed as Kaplan-Meier survival curves. The median survival times are shown in the table. The P values were determined using log-rank test. *, $P < 0.006$.

these genes, including the *ctu* gene, is significantly upregulated following infection of macrophages with *F. tularensis* Schu S4 (50). The genomic organization of *ctu* with genes involved in arginine utilization and their transcriptional upregulation following macrophage infection (50) raise the possibility that deletion of *ctu* diminishes the ability of the Δ*ctu* mutant to grow in a nutrient-limiting macrophage environment. However, arginine decarboxylase (FTT0432), an enzyme that degrades arginine into agmatine, provides an additional arginine metabolism mechanism in *Francisella* that may compensate for the loss of CTU. Our laboratory is now in the process of creating deletion mutants of additional genes involved in arginine utilization in the virulent Schu S4 strain. These mutant strains will allow us to explore further whether arginine is a major substrate that is required for intramacrophage survival of *Francisella*.

The NO produced by IFN-γ-activated murine macrophages reduces infectivity of *F. tularensis* LVS and Schu S4 (17, 27, 30, 35). Similarly, iNOS is required to resolve LVS infection in mouse models (29). Our results have shown elevated NO levels in culture supernatants from the Δ*ctu* mutant-infected macrophages (Fig. 5A). Additionally, the Δ*ctu* mutant survived similarly to the WT Schu S4 in *inos*$^{-/-}$ macrophages (Fig. 5B), suggesting an NO-dependent mechanism for killing of the Δ*ctu* mutant. In activated macrophages, increased NO levels are associated with increased citrulline generated as a result of breakdown of arginine by iNOS. The citrulline is recycled to generate arginine via an arginine-citrulline cycle in the macrophages (19). The CTU of *F. tularensis* Schu S4 degrades citrulline to ornithine and ammonia and, thus, may inhibit arginine resynthesis in the infected macrophages. However, this process might require secretion of CTU by *F. tularensis* Schu S4. The PsortB software analysis of CTU did not predict its exact subcellular localization but provided identical scores for all possible locations, including that of the secreted form (data not shown). In the absence of concrete evidence on the secretory nature of CTU, we speculate that *Francisella* depletes the arginine pool in macrophages by an active uptake and metabolism of arginine via CTU and arginine decarboxylase, thereby reducing the substrate for iNOS and subsequent NO production. Inhibiting this aspect of the innate immune response could help *Francisella* resist killing by macrophages. *Chlamydophila pneumoniae* and *Helicobacter pylori* also use a similar strategy and deplete arginine to reduce iNOS activity and NO abundance (14, 46).

It has been shown that *Francisella*, when grown in an acidic medium, causes alkalization of the pH due to generation of ammonia (7). The ammonia produced via deamination of amino acids also serves to stabilize bacterial cytoplasmic pH upon exposure to an acidic environment, such as in the phagosomal vacuoles (40). The ammonia generated by CTU has been proposed to play a role in neutralization of endosomal pH that leads to phagosomal maturation arrest (25). It has also been reported that inhibition of acidification and phagosomal maturation enhances intramacrophage survival of *Francisella* (8, 43), *Helicobacter pylori* (44) and *Mycobacterium tuberculosis* (16). On the other hand, neutralization of phagosomal pH by ammonium chloride (NH_4Cl) treatment of macrophages restores intramacrophage survival of *H. pylori* urease mutants, which are deficient for ammonia production (44). Thus, an inability to modulate the phagosomal environment or to maintain the bacterial pH homeostasis in the absence of *ctu* may also offer an explanation for attenuated intramacrophage survival of the Δ*ctu* mutant. However, NH_4Cl treatment of macrophages resulted in a modest two- to threefold increase in the survival of ingested Δ*ctu* mutant at 24 h PI (data not shown). This small improvement in survival of the Δ*ctu* mutant following NH_4Cl treatment of infected macrophages suggests that CTU alone may not cause a significant change in the phagosomal environment. Other genes like asparaginase, glutaminase, and arginine deiminase genes may still produce ammonia in the absence of CTU. The presence of these multifactorial and redundant mechanisms potentially argues in favor of our observation that the Δ*ctu* mutant was not cleared completely by the infected macrophages. It is possible that in the absence of *ctu*, these redundant mechanisms compensate for its loss.

The transcomplementation studies provided the evidence that the Δ*ctu* mutation itself is responsible for the reduced-virulence phenotype. Transcomplementation of Δ*ctu* restored virulence to the levels intermediate between the WT and the Δ*ctu* mutant phenotype in cell culture-based assays and a mouse model of respiratory tularemia. This partial, rather than full, restoration of the mutant to the WT phenotype could be attributed to plasmid loss in the absence of kanamycin selection in cellular and mouse infection models. The loss of p*ctu* in the absence of antibiotic selection pressure may have compromised the growth of the complemented mutant, resulting in an intermediate phenotype. Similar observations have been reported earlier for the transcomplemented Schu S4 mutant strain of *F. tularensis* (37).

Mice infected with the Δ*ctu* mutant showed a significantly extended median time to death compared to that of the WT Schu S4-infected mice, but all mice eventually succumbed to

infection. The results are not unexpected, as several other mutants of Schu S4 that are defective for intracellular survival have also been shown to retain virulence in mice (27, 36, 37). A possible explanation could be the existence of unidentified redundant virulence mechanisms in Schu S4 that mask the effect of a single gene deletion. In addition, due to the extremely high virulence of Schu S4, even small increases in bacterial numbers are sufficient to cause death in infected mice.

To conclude, this study provides definitive evidence that CTU activity contributes to intramacrophage survival and tularemia pathogenesis but is not the primary virulence factor of *F. tularensis* Schu S4. However, the association of the *ctu* gene with virulence may constitute a strong and rapid method for differentiation of highly virulent type A strains from less virulent or avirulent type B strains of *F. tularensis*.

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