

Iron Content Differs between *Francisella tularensis* Subspecies *tularensis* and Subspecies *holarctica* Strains and Correlates to Their Susceptibility to H₂O₂-Induced Killing^{∇†}

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***Francisella tularensis*, the causative agent of tularemia, is one of the most infectious bacterial pathogens known and is classified as a category A select agent and a facultative intracellular bacterium. Why *F. tularensis* subsp. *tularensis* causes a more severe form of tularemia than *F. tularensis* subsp. *holarctica* does is not known. In this study, we have identified prominent phenotypic differences between the subspecies, since we found that *F. tularensis* subsp. *tularensis* strains contained less iron than *F. tularensis* subsp. *holarctica* strains. Moreover, strain SCHU S4 of *F. tularensis* subsp. *tularensis* was less susceptible than FSC200 and the live vaccine strain (LVS) of *F. tularensis* subsp. *holarctica* to H₂O₂-induced killing. The activity of the H₂O₂-degrading enzyme catalase was similar between the strains, whereas the iron content affected their susceptibility to H₂O₂, since iron starvation rendered *F. tularensis* subsp. *holarctica* strains more resistant to H₂O₂. Complementing LVS with *fupA*, which encodes an important virulence factor that regulates iron uptake, reduced its iron content and increased the resistance to H₂O₂-mediated killing. By real-time PCR, it was demonstrated that FSC200 and LVS expressed higher levels of gene transcripts related to iron uptake and storage than SCHU S4 did, and this likely explained their high iron content. Together, the results suggest that *F. tularensis* subsp. *tularensis* strains have restricted iron uptake and storage, which is beneficial for their resistance to H₂O₂-induced killing. This may be an important factor for the higher virulence of this subspecies of *F. tularensis*, as reactive oxygen species, such as H₂O₂, are important bactericidal components during tularemia.**

Tularemia is an infectious disease caused by the Gram-negative bacterium *Francisella tularensis* (27). This facultative intracellular bacterium is one of the most infectious bacterial pathogens known and is classified as a category A select agent. In mice, as few as 10 CFU of *F. tularensis* subsp. *tularensis* causes a lethal infection, and this dose is also sufficient to establish tularemia in humans. *F. tularensis* subsp. *tularensis* causes a more severe form of tularemia than *F. tularensis* subsp. *holarctica* does. Although they exhibit differences in virulence, the two human pathogenic subspecies are highly similar in gene content; only 9 genes are unique to the highly virulent subspecies *F. tularensis* subsp. *tularensis*, and 20 genes are inactivated in all subspecies but *F. tularensis* subsp. *tularensis* (23). Recently, five of the unique genes were individually deleted in SCHU S4, the prototypic strain of *F. tularensis* subsp. *tularensis*, and the resulting mutants were found to retain full virulence in mice (10). Thus, the results did not indicate that the genes unique to *F. tularensis* subsp. *tularensis* contributed to its high virulence. Instead, the results imply that the relatively low virulence of *F. tularensis* subsp. *holarctica*

strains may be a combined effect of multiple missing genes or an effect of differential regulation and expression of genes present in both subspecies.

The attenuated live vaccine strain (LVS) of *F. tularensis* is a spontaneous mutant of *F. tularensis* subsp. *holarctica* that has acquired a number of genomic deletions (22). FTT0918 (*fupA*) is a major virulence factor in both *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* (24, 30). In LVS, a 252-residue carboxy-terminal sequence of this gene and a 230-residue amino-terminal sequence of the downstream gene FTT0919 (*fupB*) are deleted. The remaining residues of *fupA* and *fupB* encode a fusion protein in LVS. A recent study demonstrated that the deletion in *fupA* makes a crucial contribution to the attenuation of LVS (24). The function of *fupA* in SCHU S4, and presumably in all strains with an intact gene, is related to regulation of siderophore-dependent and -independent iron uptake (13). Since LVS carries a deletion in *fupA*, it is likely that the iron uptake and regulation are distinct from those in other *F. tularensis* strains.

Mammalian hosts respond to infection by inducing hypoferrinemia; i.e., the concentration of iron in extracellular fluids and plasma drastically decreases. This has also been demonstrated to occur during tularemia in humans (17). Since iron is essential for the growth of *F. tularensis*, the bacterium has needed to develop means to sequester iron even under such iron-limiting conditions, but how this is performed is unknown. *In vitro*, in iron-deficient medium, iron uptake depends on the siderophore rhizoferrin (4, 28). Its synthesis, export, and uptake

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are dependent on genes in the *Francisella* siderophore locus (*fslA* to *fslE*). The iron uptake and storage have to be carefully regulated to avoid the possibility that intracellular iron together with H₂O₂ generates highly toxic hydroxyl radicals and anions through the iron-driven Fenton reaction (32). In *F. tularensis*, as in most bacteria, the ferric iron uptake regulator A (FurA) is the main regulator of genes related to iron uptake (8, 19). When sufficient iron is available, FurA chelates Fe²⁺ and binds to a Fur box near the transcription start of the gene and thereby suppresses the expression. However, under iron deficiency, the absence of Fe²⁺ leads to release of Fur from the box, and thereby the gene can be transcribed. The iron uptake is further regulated by the oxidative status of the bacterial cell through the oxidative stress transcriptional regulator *oxyR* (8). In *Escherichia coli*, *oxyR* is activated by elevated H₂O₂ concentrations and induces the expression of *fur* (33). Fur then suppresses the uptake of iron as described above. Thus, *oxyR* ensures that low levels are maintained under oxidative stress to reduce the iron-driven Fenton reaction.

All organisms that live in an oxygen-rich environment have to cope with various degrees of oxidative stress, since reactive oxygen species (ROS) are by-products of the endogenous metabolism. In addition, ROS, e.g., H₂O₂, constitute one of the main defenses of the mammalian host against invading pathogens (18). *In vitro*, there is a very good correlation between resistance to H₂O₂ and the virulence of *F. tularensis* strains. SCHU S4 is more resistant than the *F. tularensis* subsp. *holarctica* strain FSC200, and LVS is the least resistant (14). The mechanism behind the high resistance of *F. tularensis* subsp. *tularensis* is not clear. In fact, there appears to be a relatively higher contribution of catalase to the virulence of LVS than to that of SCHU S4 (14). This implies that the *F. tularensis* subsp. *tularensis* subspecies of the pathogen has evolved complementary, catalase-independent mechanisms to withstand the antimicrobial effects exerted by H₂O₂. As described above, it is likely that the iron uptake in LVS is different from that in *F. tularensis* subsp. *tularensis*, and since iron potentiates the toxicity of H₂O₂ through the Fenton reaction, we asked if iron regulation is a factor that contributes to H₂O₂-mediated killing. Specifically, we investigated the capability of *F. tularensis* strains to store iron and how the different iron contents, catalase activities, and gene regulation of the bacteria influenced their resistance to H₂O₂-mediated killing.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study are listed in Table 1. *F. tularensis* LVS was originally obtained from the American Type Culture Collection (ATCC 29684). The *F. tularensis* subsp. *tularensis* strains and the *F. tularensis* subsp. *holarctica* strains were obtained from the *Francisella* Strain Collection (FSC) of the Swedish Defense Research Agency, Umeå, Sweden. All *F. tularensis* subsp. *tularensis* strains except for FSC043 and all *F. tularensis* subsp. *holarctica* strains except for LVS carry an intact *fupA* gene. To complement the LVS strain, a construct containing the *FTT0918* gene was sequenced and cloned into pDMK2 (SalI-NotI). The resulting clone was introduced into *E. coli* S17-1 *pir* and conjugated into LVS as described previously (7). The resulting mutant strain, denoted FSC697, was verified by PCR and sequencing (Eurofins MWG Operon). The *cis* complementation ensured chromosomal integration of the functional gene and expression regulated by its native promoter.

All bacteriological work was carried out in a biosafety level 3 facility certified by the Swedish Work Environment Authority and also certified to be select agent compliant by the U.S. Centers for Disease Control and Prevention (CDC).

TABLE 1. Strains used in this study

| Strain ^a | Origin or genetic feature (reference) |
|---|--|
| <i>F. tularensis</i> subsp. <i>tularensis</i> | |
| SCHU S4 | Human, USA, 1941 |
| FSC013 | Germany, 1960 |
| FSC033 | Squirrel, USA |
| FSC042 | Hare, Canada |
| FSC043 | SCHU S4 spontaneous mutant (30) |
| FSC046 | Human, USA, 1940 |
| FSC053 | Human ulcer |
| FSC054 | Rabbit, USA, 1953 |
| FSC198 | Slovakia, 1988 |
| FSC199 | Slovakia, 1988 |
| FSC604 | USA, 1959 |
| <i>F. tularensis</i> subsp. <i>holarctica</i> | |
| FSC200 | Human ulcer |
| LVS | ATCC 29684 |
| FSC697 | LVS expressing <i>FTT0918</i> in <i>cis</i> (this study) |
| FSC076 | Hare, Sweden, 1981 |
| FSC089 | Human, Norway, 1989 |
| FSC157 | Human, Sweden, 1994 |
| FSC226 | Human ulcer, Ljusdal, Sweden, 1998 |
| FSC244 | Human, Sweden, 1995 |
| FSC663 | Human, Sweden |
| FSC812 | Human, Sweden |
| FSC831 | Human, Sweden |

^a All *Francisella* strains were from the FSC, FOI Swedish Defense Research Agency, Umeå, Sweden, and the strain collection number is indicated.

Preparation of growth media. Chamberlain's defined medium (CDM) (2) iron depleted by chelation (C-CDM) was produced as described previously (3, 13). Briefly, 1% (wt/vol) Chelex-100 (Bio-Rad, Hercules, CA) was added to CDM without FeSO₄, and the mixture was kept in rotation for 24 h at 4°C. The Chelex-100 was removed by filtering the medium through a 0.2-μm-pore-size Millipore filter (Biosciences, Stockholm, Sweden), and the chelating step was repeated once. The medium was thereafter supplemented with essential cations (MgSO₄, 0.55 mM; ZnCl₂, 1.4 μM; CuSO₄, 0.2 μM; MnCl₂, 1.0 μM; CaCl₂, 5 μM). Sterility was ensured by a second filtration.

Deferoxamine (DFO) is an iron chelator and was used to prepare agar plates devoid of free iron in the medium (DFO plates). These plates were composed of 1 part of 4% agar GC medium base (BD Diagnostic Systems, Sparks, MD), 1 part of C-CDM, and 25 μg/ml DFO (Sigma-Aldrich, St. Louis, MO).

McLeod agar plates (MC plates) were composed of 1% (wt/vol) hemoglobin (Oxoid Ltd., Hampshire, England), 3.6% (wt/vol) GC agar base (BD Diagnostic Systems), and 1% (vol/vol) IsoVitalEX (BD Diagnostic Systems).

Ferrozine assay. A ferrozine-based method was used to measure the total amount of iron in the bacterial samples (21). Ferrozine forms a complex with Fe²⁺ that absorbs strongly at 562 nm. Bacteria cultivated overnight on MC plates or DFO plates were resuspended to an A₆₀₀ of 1.0 in phosphate-buffered saline (PBS). One milliliter of the bacterial suspension was centrifuged at 13,000 rpm for 5 min, and the resulting bacterial pellet was lysed with 100 μl of 50 mM NaOH. The solution was mixed thoroughly to ensure complete lysis of the bacteria. One hundred microliters of 10 mM HCl was added to the lysate. To release protein-bound iron, the samples were treated with 100 μl of a freshly prepared solution of 0.7 M HCl and 2.25% (wt/vol) KMnO₄ in H₂O and incubated for 2 h at 60°C. All chemicals used were from Sigma-Aldrich. Thereafter, the samples were mixed with 100 μl of the iron detection reagent, composed of 6.5 mM ferrozine, 6.5 mM neocuproine, 2.5 M ammonium acetate, and 1.0 M ascorbic acid dissolved in water. The samples were incubated for 30 min, and insoluble particles were removed by centrifugation. Two hundred microliters of the supernatant was transferred to a 96-well plate, and the A₅₆₂ was determined in a microplate reader (Paradigm; Beckman Coulter, Bromma, Sweden). The iron content of the sample was calculated by comparing its absorbance to that of a range of samples with FeCl₃ concentrations in the range of 0 to 40 μM that had been prepared identically to the test samples. The correlation coefficients of the

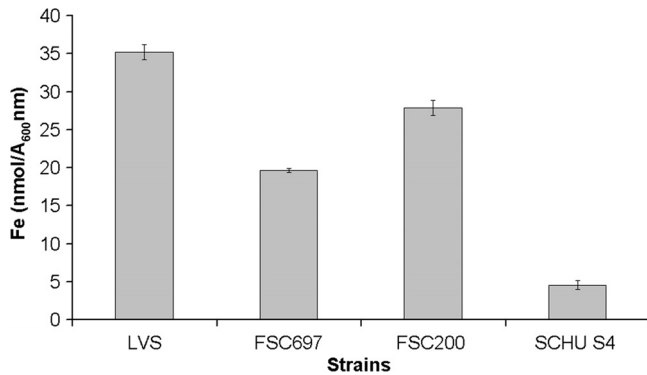


FIG. 1. Iron content of *F. tularensis* strains cultivated on MC plates. The bars represent the averages of at least 12 independent observations. The error bars show the standard errors of the means.

standard curves varied from 0.998 to 0.999. The detection limit of the assay was 1 μM Fe. The intrasample variations (i.e., variations among samples from the same culture) were less than 0.3 nmol/ 3×10^9 bacteria.

H₂O₂ susceptibility assay. Bacteria cultivated overnight on MC plates were resuspended and diluted in PBS to an estimated 3×10^6 CFU/ml. The bacterial suspension of each *F. tularensis* strain was divided into test tubes with H₂O₂ added or control tubes that were untreated. The tubes were incubated for 4 h at room temperature without shaking. Thereafter, the samples were serially diluted and spread on MC plates that were incubated at 37°C in 5% CO₂ for 3 days before enumeration of the CFU. The data are expressed as the percent survival of a strain exposed to H₂O₂ relative to the viability of that strain in the control tube not exposed to H₂O₂.

Catalase assay. Bacteria cultivated overnight on MC plates or DFO plates were resuspended to an A₆₀₀ of 1.9 in PBS. Bacteria from 1 ml of the suspension were collected by centrifugation at 13,000 rpm for 5 min. Protein extracts were prepared from the bacterial pellet using 300 μl of B-PER bacterial protein extraction buffer (Thermo Scientific, Rockford, IL). No DNase or lysozyme was supplied to the lysis buffer. The bacteria were mixed thoroughly in the buffer and left for 10 min on the bench to allow complete disruption of the bacteria. Thereafter, the lysate was centrifuged for 3 min to remove any cell debris. The protein concentration in the extracts was determined using the Bradford reagent, according to the manufacturer's instruction (Fermentas, St. Leon-Rot, Germany).

The catalase assay was performed in a 96-well plate by mixing 110 μl of PBS with 10 μl of the protein extract. Then, 80 μl of H₂O₂ (100 mM) was supplied to start the reaction. The decomposition of H₂O₂ was measured by monitoring the decrease in absorbance at 240 nm using a microplate reader (Paradigm; Beckman Coulter). The initial linear portion of the curve was used to calculate the change in the A₂₄₀. A molar extinction coefficient (ϵ) of H₂O₂ at 240 nm of 43.6 M⁻¹cm⁻¹ was used to calculate the concentration of H₂O₂ using the Beer-Lambert law, $A = \epsilon cl$, where A is absorbance, c is the molar concentration, and l is the path length. One unit of catalase was defined as the amount that decomposes 1 μmol of H₂O₂ per minute at 25°C per mg protein. In total four (LVS and FSC697) or eight (SCHU S4 and FSC200) protein extracts were analyzed per strain and growth condition.

Analysis of gene expression. Bacteria cultivated overnight on MC plates were resuspended to an optical density at 600 nm of 1.0 in PBS, and 1 ml of the bacterial suspension was centrifuged at 13,000 rpm for 5 min. RNA was extracted from the bacterial pellet using Trizol, according to the manufacturer's protocol (Invitrogen). cDNA was synthesized from this RNA, and quantitative real-time PCR (RT-PCR) was used to analyze the cDNA samples. In order to remove contaminating DNA, the RNA samples were treated with DNase (DNA-free kit; Ambion, Inc., Austin, TX) in accordance with the protocol supplied by the manufacturer. The RNA was quantified by use of a Nanodrop device (Thermo Fisher Scientific, Wilmington, DE). cDNA was synthesized from 1 μg of the extracted RNA using RNAscript reagent (Bio-Rad), according to the protocol provided by the manufacturer. A negative control was prepared by excluding the enzyme from the cDNA synthesis. In order to degrade any remaining RNA, the cDNA was treated with 2.0 μl of 2.5 M NaOH at 42°C for 10 min, after which the pH was adjusted by the addition of 5 μl of 1 M HCl. The samples were thereafter diluted and stored at -20°C.

RT-PCR was performed in an ABI Prism 7900HT sequence detection system

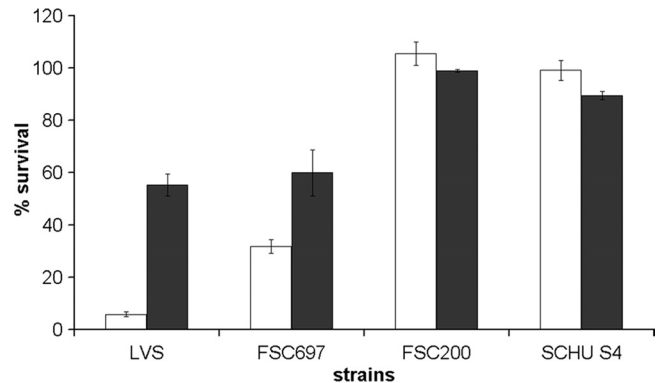


FIG. 2. Susceptibility to H₂O₂ of *F. tularensis* strains cultivated on MC plates (iron replete, white bars) or plates containing the iron chelator DFO (iron deplete, black bars). These bacteria were resuspended in PBS to a concentration of 3×10^6 CFU/ml and exposed to 1 mM H₂O₂ for 4 h at room temperature. The bars represent the averages of at least three independent experiments with three observations in each one. The error bars show the standard errors of the means.

(Applied Biosystems, Foster City, CA) using a SYBR green I PCR kit (Applied Biosystems), as recommended by the manufacturer. Each reaction mixture contained 12.5 μl of the SYBR green mix, 250 nM forward and reverse primers, and 5 μl of cDNA, and the total volume was adjusted to 25 μl with water. Forward and reverse primers were obtained from Invitrogen, and their sequences are listed in Table S1 in the supplemental material. The reactions were performed in a MicroAmp 96-well plate (Applied Biosystems) capped with MicroAmp optical adhesive seal. The reaction mixtures were incubated at 50°C for 2 min and 10 min at 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C. The final cycle consisted of incubation at 95°C for 15 s, 60°C for 15 s, and 95°C for 15 s. The threshold cycle (C_T) values of selected genes were normalized to the C_T value of the housekeeping gene *polA* (28). The relative gene expressions were calculated as described by Gavrilin et al. (6). Normalized C_T values were used for statistical evaluation of the data.

Statistical analysis. For statistical evaluation, one-tailed Student's t test in the statistical software program SPSS was used.

RESULTS

Bacterial iron content. SCHU S4, FSC200, LVS, and LVS complemented with *fupA*, FSC697, were cultivated on MC plates, and iron contents were determined by use of the ferrozine assay. FSC200 contained, on average, 6-fold more iron than SCHU S4 ($P < 0.001$) (Fig. 1) but less iron than LVS ($P < 0.001$). FSC697, LVS expressing *fupA* in *cis*, contained less iron than both LVS and FSC200 ($P < 0.001$) but more iron than SCHU S4 ($P < 0.001$) (Fig. 1). All strains grown on agar plates with DFO had an iron content lower than the detection limit of the assay, 0.2 nmol. Henceforth, strains grown on MC plates and DFO plates are denoted iron replete and iron deplete, respectively.

Resistance to H₂O₂-mediated killing. Iron in combination with H₂O₂ generates highly reactive hydroxyl radicals and anions through the Fenton reaction. In view of this, we determined if the iron content of the bacteria affected their susceptibility to H₂O₂. Iron-replete and iron-deplete SCHU S4, FSC200, LVS, and FSC697 were exposed to 1 mM H₂O₂ for 4.0 h. Iron-replete FSC697 survived significantly better ($P < 0.001$) than LVS (Fig. 2). In contrast, iron-deplete LVS and FSC697 survived the H₂O₂ challenge equally well and significantly better ($P < 0.001$) than they did in the iron-replete state.

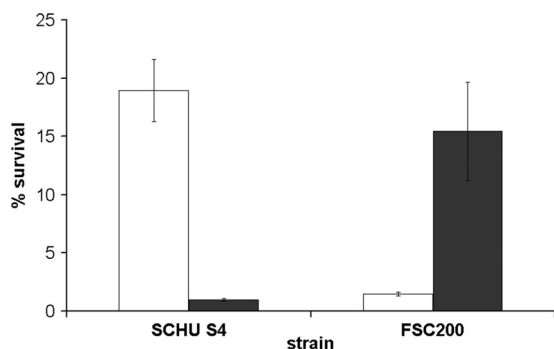


FIG. 3. Susceptibility to H₂O₂ of *F. tularensis* strains cultivated on MC plates (iron replete, white bars) or plates with the iron chelator DFO (iron deplete, black bars). These bacteria were resuspended in PBS to a concentration of 3×10^6 CFU/ml and exposed to 6 mM H₂O₂ for 4 h at room temperature. The bars represent the averages of two independent experiments with three observations in each one. The error bars show the standard errors of the means.

The viability of SCHU S4 and FSC200 was not affected by 1 mM H₂O₂ (Fig. 2), so they were therefore exposed to 6 mM H₂O₂ (Fig. 3). Iron-replete SCHU S4 survived the 6 mM H₂O₂ challenge significantly better than did FSC200 ($P < 0.02$) (Fig. 3). Similar to LVS and FSC697, FSC200 became more resistant to H₂O₂ when it was depleted of iron ($P < 0.02$). Notably, iron-deplete FSC200 survived the H₂O₂ challenge as well as iron-replete SCHU S4 did. In contrast to the other strains tested, the H₂O₂-mediated killing of SCHU S4 increased dramatically when it was depleted of iron relative to the level of killing in the iron-replete state (Fig. 3). LVS and FSC697 were rapidly eradicated when they were exposed to 6 mM H₂O₂ (data not shown).

In summary, strains of *F. tularensis* subsp. *holarctica* origin (FSC200, LVS, and FSC697) contained significantly more iron than SCHU S4 and were more susceptible to H₂O₂-mediated killing. These strains became more resistant to H₂O₂-mediated killing when their iron content was lowered. Notably, FSC697 contained less iron than LVS and was more resistant to H₂O₂-mediated killing. Thus, a low intracellular iron concentration appeared to increase the resistance of *F. tularensis* subsp. *holarctica* strains to H₂O₂-mediated killing.

Iron contents of *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* strains. To establish if the relatively low iron content of SCHU S4 and high iron content of FSC200 are typical of other strains belonging to the respective subspecies, the iron contents of 11 *F. tularensis* subsp. *tularensis* strains and 9 *F. tularensis* subsp. *holarctica* strains were measured by the ferrozine assay. All *F. tularensis* subsp. *tularensis* strains tested contained less than 8 nmol iron, and all *F. tularensis* subsp. *holarctica* strains tested contained more than 18 nmol iron (Fig. 4). Thus, in general, *F. tularensis* subsp. *tularensis* strains contained less iron than *F. tularensis* subsp. *holarctica* strains after cultivation on iron-rich medium. Noteworthy, this was true also for FSC043, a spontaneous SCHU S4 mutant that contains the same fusion of *fupA* and *fupB* as LVS (26, 30).

Expression of genes related to iron uptake and storage. We hypothesized that the difference in iron content of strains of the two subspecies may be due to differential expression of genes related to iron uptake and storage. The relative mRNA

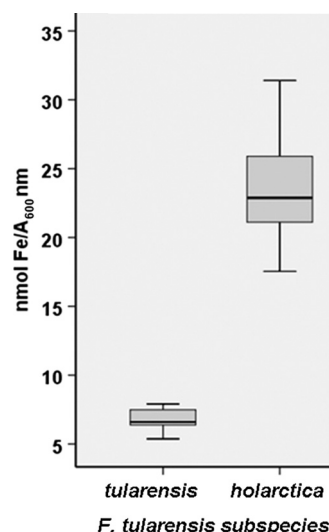


FIG. 4. Iron content of *F. tularensis* strains cultivated on MC plates. The bars represent the averages of three independent observations. The line through each box shows the median, with quartiles being at either end of each box. The vertical lines indicate maximum and minimum values.

copy numbers of ferrous uptake proteins A and B (*feoA* and *feoB*), ferric uptake protein A (*fupA*), ferric uptake regulator A (*furA*), the iron storage ferritin (*fn*), and genes of the *fsl* operon (*fslA* to *fslG*) in the strains were measured by real-time PCR. FSC200, LVS, and FSC697 expressed significantly higher levels of *fslA* than did SCHU S4 (Table 2). In addition, *fslD* was upregulated in FSC200 relative to the level of expression in SCHU S4. *fslE* and *fslF* were significantly downregulated in LVS and FSC697 relative to the levels of expression in SCHU S4, and *fslF* was also significantly downregulated in FSC200. The *fn* copy number was significantly elevated in all strains of *F. tularensis* subsp. *holarctica* relative to the level of expression in SCHU S4. All other genes tested were expressed at similar levels in the *F. tularensis* strains.

TABLE 2. Real-time PCR analysis of the expression of genes related to iron uptake or storage

| Gene | RCN ^a | | | |
|-------------|-----------------------|--------------------------|------------------------|--------------------------|
| | SCHU S4 | FSC200 | LVS | FSC697 |
| <i>fslA</i> | 381 ± 87 ^b | 1,100 ± 147 ^b | 987 ± 205 ^c | 1,214 ± 184 ^b |
| <i>fslB</i> | 193 ± 49 | 291 ± 64 | 94 ± 17 | 161 ± 40 |
| <i>fslC</i> | 121 ± 39 | 171 ± 8 | 75 ± 17 | 138 ± 38 |
| <i>fslD</i> | 37 ± 6 | 78 ± 19 ^c | 27 ± 5 | 53 ± 8 |
| <i>fslE</i> | 57 ± 20 | 28 ± 8 | 12 ± 5 ^c | 18 ± 14 ^c |
| <i>fslF</i> | 153 ± 17 | 47 ± 17 ^c | 29 ± 11 ^b | 16 ± 5 ^d |
| <i>feoA</i> | 26 ± 6 | 27 ± 2 | 36 ± 6 | 45 ± 7 |
| <i>feoB</i> | 17 ± 10 | 18 ± 8 | 8 ± 3 | 11 ± 5 |
| <i>fur</i> | 243 ± 59 | 95 ± 19 | 167 ± 42 | 123 ± 36 |
| <i>fupA</i> | 351 ± 129 | 149 ± 73 | 341 ± 94 | 72 ± 3 |
| <i>fn</i> | 53 ± 7 | 123 ± 11 ^b | 139 ± 29 ^c | 225 ± 48 ^b |

^a Copy number of respective gene relative to the housekeeping gene *FTT0111*. The average of the relative copy numbers obtained from three to five cDNA samples prepared on different days and standard error of the mean are shown.

^b $P < 0.01$ relative to SCHU S4.

^c $P < 0.05$ relative to SCHU S4.

^d $P < 0.001$ relative to SCHU S4.

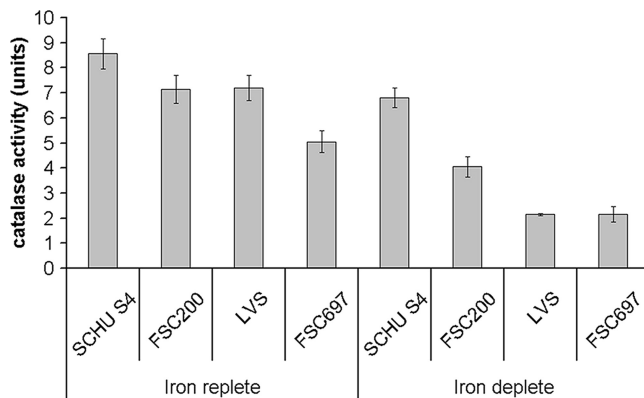


FIG. 5. Catalase activity in protein extracts from *F. tularensis* strains cultivated on MC plates (iron replete) or on plates with DFO (iron deplete). The bars represent the averages of four to eight independent experiments. The error bars show the standard errors of the means.

Genes related to defense against oxidative stress, *oxyR*, *katG*, *gpx*, *ahpC1*, *sodB*, *sodC*, *trxA1*, *trxB*, *gshA*, *gshB*, *grxA*, *grxB*, *msrA1*, and *msrA2*, were also analyzed by real-time PCR, but there were no significant differences in expression levels between the *F. tularensis* strains (data not shown).

Catalase activity. Catalase contributes to the resistance of SCHU S4 and LVS to H_2O_2 (14), but the role of catalase in FSC200 has not been investigated. The catalase activity of the strains was measured to determine if there were any differences that could be correlated to their susceptibility to H_2O_2 . Moreover, as catalase is a heme-containing enzyme, we wanted to establish if the iron content of the bacteria affected their catalase activity. The activity was measured in protein extracts from iron-deplete and iron-replete strains SCHU S4, FSC200, LVS and FSC697 (Fig. 5). The catalase activities of SCHU S4, FSC200, and LVS were similar under iron-replete conditions, whereas the activity in FSC697 was lower than that in SCHU S4 ($P < 0.001$) and LVS ($P < 0.05$) under iron-replete conditions. Depletion of the iron pool reduced the catalase activity of each of the strains in relation to the activity under iron-replete conditions ($P < 0.05$ for SCHU S4; $P < 0.001$ for FSC200 and LVS; $P < 0.01$ for FSC697). The decrease was most pronounced in the *F. tularensis* subsp. *holarctica* strains, and the catalase activity of iron-depleted SCHU S4 thereby was significantly higher than that of the other strains ($P < 0.001$). Protein extracts from SCHU S4 $\Delta katG$ and LVS $\Delta katG$ mutants did not decompose H_2O_2 , thereby validating the specificity of the assay (data not shown).

DISCUSSION

The human vaccine strain LVS is highly virulent in mice and causes a tularemia-like disease. A multitude of studies with LVS in the mouse model have delineated host protective mechanisms against *F. tularensis*. It has been found that gamma interferon, tumor necrosis factor alpha, CD4 and CD8 T cells, neutrophils, and the inducible nitric oxide synthase all make important contributions to the host defense (5). In addition, the phagocyte oxidase (phox) is critical to restrict bacterial replication during the innate phase of the infection (15).

Phox is a major source of superoxide (O_2^-) production (18) and the degradation product H_2O_2 ; therefore, it is not surprising that catalase, which degrades H_2O_2 , is an important virulence factor of LVS (14). In contrast, catalase makes only a minor contribution to the virulence of SCHU S4. This implies that virulent strains of *F. tularensis*, unlike LVS, possess additional strategies to avoid H_2O_2 -mediated killing. Our results identify iron regulation as a factor that determines the susceptibility of *F. tularensis* to H_2O_2 -mediated killing and also demonstrate that there is a correlation between iron content and virulence. A major finding was that *F. tularensis* subsp. *tularensis* strains contained less iron than *F. tularensis* subsp. *holarctica* after growth on iron-rich medium. In line with this, the expression of *ftn*, encoding the iron storage protein ferritin, was expressed at higher levels in FSC200 (*F. tularensis* subsp. *holarctica*) than in SCHU S4 (*F. tularensis* subsp. *tularensis*). Despite the high iron content in FSC200, genes of the *fsl* operon (*fslA* and *fslD*), which are negatively regulated by FurA, were expressed at higher levels in FSC200 than in SCHU S4. A similar regulation of *ftn* and *fslA* was apparent in FSC200 and LVS. In contrast to *fslA*, *fslE* was expressed at lower levels in LVS than in SCHU S4. A reason for this may be that in LVS induction of *fslE* requires more stringent iron limitation than does induction of *fslA* (4, 11). It has previously been observed that ferritin exists in different isoforms and that some of these are unique to each *F. tularensis* subspecies (9, 12). Together with these reports, our results demonstrate that the fine-tuning of Fur regulation and the regulation of iron uptake and storage are fundamentally different between the two subspecies of *F. tularensis*.

FSC697, LVS expressing the full-length *fupA* gene, contained even less iron than FSC200 and was almost as virulent as FSC200 (unpublished data), in accordance with recent findings on another *fupA*-complemented LVS strain (24). Thus, the partial deletion of *fupA* in LVS is a major factor for its attenuation, and the reason is likely related to changes in iron metabolism, since it is the only function attributed to the protein. In *F. tularensis* subsp. *tularensis*, FslE is the siderophore receptor and FupA influences the expression of the *fsl* operon (13, 19). In contrast, the hybrid protein FupA/FupB functions as the siderophore receptor in LVS, whereas FslE has a minor role in the process (25). We have observed that FSC697, in which the hybrid gene is missing, could still utilize siderophores (unpublished data). This suggests either that FupA functions as a siderophore receptor when it is expressed in LVS or that in the presence of FupA, FslE functions as a receptor for the siderophore. The last scenario is in accordance with the finding that both FupA and FslE are required by *F. tularensis* subsp. *tularensis* to utilize siderophores (13, 19). Notably, disrupting the siderophore system in SCHU S4 had no marked effect on its virulence (13), indicating that FupA must have a siderophore-independent function that is critical for virulence.

Introduction of *fupA* into LVS (FSC697) reduced the iron content below the level found in *F. tularensis* subsp. *holarctica* strains. If FupA was directly responsible for the low level of iron in *F. tularensis* subsp. *tularensis*, deletion of the gene should result in increased iron content. However, the spontaneous SCHU S4 mutant FSC043, which contains the same FupA-FupB deletion as LVS (26, 30), did not demonstrate an

elevated iron content. Moreover, the protein is identical in virulent strains of the two subspecies. Thus, although FupA has an important role in the iron regulation of *F. tularensis* strains in general, other, subspecies-specific factors play a more important role for the regulation of the total iron levels. This implies that FupA in *F. tularensis* subsp. *tularensis* operates coordinately with genes not present in *F. tularensis* subsp. *holarctica* or that these genes are differentially regulated. Together, the results illustrate the complexity of the regulatory network that controls the iron homeostasis in *F. tularensis*. FupA appears to be one of the important components of this network.

Our findings point to a strong correlation between the iron content of the *F. tularensis* subsp. *holarctica* strains and their susceptibility to H₂O₂. The first evidence was the increased resistance of LVS and FSC200 to H₂O₂-mediated killing after iron depletion. The second evidence was that introduction of *fupA* into LVS reduced the iron content and increased the resistance to H₂O₂-mediated killing. The catalytic activity of catalase is dependent on iron (31), so it was not surprising that the catalase activity decreased in the iron-depleted strains. However, catalase is critically required for LVS to withstand H₂O₂-mediated killing, so an increased resistance to H₂O₂ paralleled by a reduced catalase activity appears to be paradoxical (14). Rather, the results illustrate how detrimental excess iron may be for bacteria when they are exposed to H₂O₂. The iron-driven Fenton reaction probably decreased when the iron content was reduced, and therefore, the bacteria survived the H₂O₂ challenge better. This is the first study to demonstrate that the level of iron modifies the susceptibility of *F. tularensis* to H₂O₂. The important role of iron to modulate the adaptation to oxidative stress in other bacteria is well documented. For example, and similar to the results with LVS and FSC200, *Staphylococcus aureus* (20) and *Escherichia coli* (1) grown under iron-rich conditions demonstrated increased susceptibility to H₂O₂ compared to that of bacteria grown under iron-limiting conditions. In addition, inactivation of the *fur* gene (resulting in deregulation of iron metabolism) increases susceptibility to oxidative stress. This effect can be reversed by iron chelation or introducing a *tonB* mutation, thereby blocking iron uptake (16, 29).

The correlation between iron and the H₂O₂ susceptibility of the *F. tularensis* subsp. *holarctica* strains implies that the natural, low level of iron in strains of *F. tularensis* subsp. *tularensis* is an important trait required to withstand H₂O₂-mediated damage upon infection. Nevertheless, the resistance of SCHU S4 to H₂O₂ was highly dependent on iron. In contrast to LVS and FSC200, the susceptibility of SCHU S4 increased dramatically when it was in the iron-deplete versus the iron-replete state. Thus, the mechanism(s) behind the defense against H₂O₂-mediated damage in SCHU S4 is dependent on iron. Catalase is an important enzyme for SCHU S4 to withstand H₂O₂, but its activity was only slightly affected by iron depletion, so another, not yet identified factor(s) in SCHU S4 that is dependent on iron to protect the bacterium against H₂O₂ appears to exist.

Altogether the data presented show that the regulation of iron uptake and storage is different between *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* and that iron is a factor that determines the susceptibility of the bacterium to

H₂O₂. During infection, *F. tularensis* has to cope with both iron deficiency and oxidative stress. Thus, the different handling of iron regulation and resistance to H₂O₂ by the two subspecies may be central to the differences in their virulence.

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