Kdo Hydrolase Is Required for Francisella tularensis Virulence and Evasion of TLR2-Mediated Innate Immunity

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Kdo Hydrolase Is Required for *Francisella tularensis* Virulence and Evasion of TLR2-Mediated Innate Immunity

Nihal A. Okan, Sabina Chalabaev, Tae-Hyun Kim, Avner Fink, Robin A. Ross, Dennis L. Kasper

Department of Microbiology and Immunobiology, Harvard Medical School, Boston, Massachusetts, USA

N.A.O. and S.C. contributed equally in this work.

**ABSTRACT** The highly virulent *Francisella tularensis* subsp. *tularensis* has been classified as a category A bioterrorism agent. A live vaccine strain (LVS) has been developed but remains unlicensed in the United States because of an incomplete understanding of its attenuation. Lipopolysaccharide (LPS) modification is a common strategy employed by bacterial pathogens to avoid innate immunity. A novel modification enzyme has recently been identified in *F. tularensis* and *Helicobacter pylori*. This enzyme, a two-component Kdo (3-deoxy-β-manno-octulosonic acid) hydrolase, catalyzes the removal of a side chain Kdo sugar from LPS precursors. The biological significance of this modification has not yet been studied. To address the role of the two-component Kdo hydrolase KdhAB in *F. tularensis* pathogenesis, a ΔkdhAB deletion mutant was constructed from the LVS strain. In intranasal infection of mice, the ΔkdhAB mutant strain had a 50% lethal dose (LD50) 2 log10 units higher than that of the parental LVS strain. The levels of the proinflammatory cytokines tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β) in bronchoalveolar lavage fluid were significantly higher (2-fold) in mice infected with the ΔkdhAB mutant than in mice infected with LVS. *In vitro* stimulation of bone marrow-derived macrophages with the ΔkdhAB mutant induced higher levels of TNF-α and IL-1β in a TLR2-dependent manner. In addition, TLR2−/− mice were more susceptible than wild-type mice to ΔkdhAB bacterial infection. Finally, immunization of mice with ΔkdhAB bacteria elicited a high level of protection against the highly virulent *F. tularensis* subsp. *tularensis* strain Schu S4. These findings suggest an important role for the *Francisella* Kdo hydrolase system in virulence and offer a novel mutant as a candidate vaccine.

**IMPORTANCE** The first line of defense against a bacterial pathogen is innate immunity, which slows the progress of infection and allows time for adaptive immunity to develop. Some bacterial pathogens, such as *Francisella tularensis*, suppress the early innate immune response, killing the host before adaptive immunity can mature. To avoid an innate immune response, *F. tularensis* enzymatically modifies its lipopolysaccharide (LPS). A novel LPS modification—Kdo (3-deoxy-β-manno-octulosonic acid) saccharide removal—has recently been reported in *F. tularensis*. We found that the ΔkdhAB mutant was significantly attenuated in mice. Additionally, the mutant strain induced an early innate immune response in mice both *in vitro* and *in vivo*. Immunization of mice with ΔkdhAB bacteria elicited a high level of protection against the highly virulent *F. tularensis* strain Schu S4. Thus, our study has identified a novel LPS modification important for microbial virulence. A mutant lacking this modification may be used as a live attenuated vaccine against tularemia.

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*Francisella tularensis*, one of the deadliest respiratory pathogens in existence, is considered a potential biological weapon because it is readily aerosolized and exhibits a high degree of infectivity and lethality in humans. An attenuated live vaccine strain (LVS) has been developed but has not been licensed as a vaccine because of an incomplete understanding of the basis for its attenuated virulence and associated side effects. There has been a widespread research effort aimed at elucidating *Francisella* pathogenesis and identifying components for rational vaccine design. *F. tularensis* is a successful pathogen that can evade and suppress innate immune responses. After intranasal infection of mice, *F. tularensis* initially colonizes the lung, infecting several cell types but failing to stimulate proinflammatory cytokine production for the first 2 to 3 days (1–5). This initial delay in inflammation contributes to the ability of *F. tularensis* to replicate in the lung, spread into systemic organs, and cause sepsis leading to the host’s death (4). To suppress the early proinflammatory cytokine response, *F. tularensis* employs a variety of strategies, such as avoiding disclosure of itself to the innate immune system (6, 7).

Lipopolysaccharide (LPS) of *F. tularensis* plays a significant role in evasion of host immune responses (8–11). LPS, the major component of Gram-negative bacterial outer membranes (12, 13), itself has three components: (i) lipid A, a glucosamine-based glycolipid; (ii) a core oligosaccharide ketosidically linked to lipid A by an eight-carbon sugar, 3-deoxy-β-manno-octulosonic acid (Kdo); and (iii) the O-antigen polysaccharide comprising multi-
ple repeating units of a complex tetrasaccharide. The lipid A of many bacterial species such as *Escherichia coli* is a powerful stimulant of Toll-like receptor 4 (TLR4)-mediated innate immune responses. In contrast, *F. tularensis* lipid A triggers no TLR-mediated innate response because of a number of key structural modifications (9); specifically, despite initial similarity to *E. coli* lipid A, the lipid A precursor of *F. tularensis* undergoes dephosphorylation, deacylation, and carbohydrate incorporation that render the molecule undetectable by the TLR4 receptor. Differences in the length of the fatty acid chain of lipid A (16 to 18 carbons for *F. tularensis* as opposed to 12 to 14 carbons for *E. coli*) further enhance the ability of *F. tularensis* to evade TLR4-mediated innate immunity. These modifications contribute to bacterial virulence (8, 10).

A novel LPS-modifying two-component KdhAB Kdo hydrolase enzyme complex has recently been identified in *F. tularensis* (14, 15). A core LPS precursor containing two Kdo residues is first synthesized. One Kdo in the backbone of the core polysaccharide is ketosidically linked to lipid A and glycosidically linked to the remainder of the core polysaccharide, and the second Kdo residue is glycosidically attached as a side chain. KdhAB removes the side chain Kdo, leaving an LPS molecule containing only one Kdo residue (Fig. 1) (14–16). The KdhAB Kdo hydrolase is a two-component enzyme composed of a sialidase-like protein (the catalytic subunit) and a small inner membrane protein (perhaps a membrane-anchoring subunit) (15). This novel enzyme complex has been reported in two other pathogens, *Helicobacter pylori* and *Legionella pneumophila* (14, 17, 18). Its biological significance—in particular, its role in pathogenicity—has not yet been investigated. Here, we show that the *F. tularensis* ΔkdhAB LVS mutant is attenuated in a mouse model of infection and stimulates—in a TLR2-dependent manner—a stronger proinflammatory cytokine response than the parental LVS. Furthermore, immunization of mice with the ΔkdhAB mutant provides significant protection against fully virulent *F. tularensis* type A strain Schu S4.

**RESULTS**

The *F. tularensis* LVS ΔkdhAB mutant is attenuated in mice. To fully evaluate the importance of the KdhAB Kdo hydrolase in the virulence of *F. tularensis* LVS, *kdhA* and *kdhB* genes were deleted by homologous recombination in an LVS background, giving rise to the ΔkdhAB mutant. Previously described analysis by high-performance anion-exchange chromatography (HPAEC) (14) confirmed that, unlike the LPS of parental LVS, which has a single Kdo residue, the LPS of the ΔkdhAB mutant has two Kdo residues (Fig. 1; see Table S1 in the supplemental material).

To evaluate the role of Kdo hydrolase activity in *F. tularensis* virulence, we infected BALB/cByJ mice with graded doses of LVS or the ΔkdhAB mutant via the intranasal route. All mice challenged with 10^3 CFU of LVS succumbed to the infection within 9 days (Fig. 2A). In contrast, all mice challenged with a similar dose of the ΔkdhAB mutant survived (Fig. 2A). At a dose of 10^5 CFU of the ΔkdhAB mutant, 17% of the infected mice survived (Fig. 2A).

The observed phenotypes in the ΔkdhAB mutant were successfully complemented with the pkdhb plasmid carrying the *kdhA* and *kdhB* genes. LPS analysis showed that the complemented strain had only a single Kdo residue in its core LPS (see Table S1 in the supplemental material). In addition, complementation of ΔkdhAB restored virulence to the wild-type (WT) level. The survival rates of BALB/cByJ mice (10 mice in each group) 28 days after an intranasal challenge with 5 × 10^5 *F. tularensis* LVS or a ΔkdhAB mutant harboring either a vector control (pFNLT6P) or a plasmid carrying *kdhA* genes (pFdhp) were as follows: 0% for mice infected with LVS, 100% for mice infected with the ΔkdhAB mutant carrying pFNLT6P, and 20% for mice infected with the ΔkdhAB mutant carrying pFdhp. These data indicate that Kdo hydrolase activity is important for the virulence of *F. tularensis* LVS.

We also examined the ability of the ΔkdhAB mutant to colonize the lung and to disseminate into other organs after intranasal challenge of mice. Six days after infection with 10^7 bacteria, both parental LVS and its ΔkdhAB mutant had reached a similar level of colonization in the lungs, with counts of 3 × 10^8 CFU/g and 10^7 CFU/g, respectively (Fig. 2B). However, the numbers of ΔkdhAB mutant bacteria in spleen, liver, and blood were 1.5 to 3 logarithmic units lower than the numbers of LVS bacteria (Fig. 2B) (Student’s *t* test, *P* < 0.05). Overall, these results indicate that the ΔkdhAB mutant is deficient in its ability to disseminate systemically.

To determine whether the observed defect in dissemination is related to bacterial growth kinetics, growth rates were compared under different conditions. There was no difference in growth between LVS and the ΔkdhAB mutant when bacteria were cultured in broth medium or in J774A.1 macrophage-like and MH-S alveolar macrophage-like cell lines (see Fig. S1A and S1B in the supplemental material). Moreover, the ΔkdhAB mutant was not sensitive to complement-mediated killing (Fig. S1C). Therefore, the defect of the ΔkdhAB mutant in dissemination to systemic organs was not due to a growth defect or to serum sensitivity.

The cytokine response to the *F. tularensis* LVS ΔkdhAB mutant is enhanced. Within the first day after infection, mutants of *F. tularensis* that cannot modify their lipid A elicit a stronger proinflammatory response than parental LVS did (8, 19). Since the KdhAB hydrolase is involved in the modification of the LPS core oligosaccharide but not in the modification of lipid A, we compared the proinflammatory responses to LVS and the ΔkdhAB mutant. To this end, mice were challenged intranasally with 10^4 CFU of either LVS or the ΔkdhAB mutant, and the levels of tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β) in samples of bronchoalveolar lavage (BAL) fluid were determined in an enzyme-linked immunosorbent assay (ELISA). Consistent with previous reports (6), the levels of TNF-α and IL-1β in...
Characterization of \textit{kdhAB} in \textit{Francisella tularensis}

To further investigate the mechanism for enhanced TLR2 signaling by the \textit{\textit{kdhAB}} mutant, we evaluated bacterial membrane integrity. A leaky cell membrane caused by \textit{\textit{kdhAB}} mutation would potentially release TLR2 ligands into the culture supernatant to be detected by host cells. Thus, the growth behaviors of \textit{LVS} and the \textit{\textit{kdhAB}} mutant were tested in the presence of SDS and EDTA, agents that inhibit growth of bacterial cells with defective outer membranes. The similar growth characteristics of the two
FIG 3  Enhanced proinflammatory cytokine response to the F. tularensis LVS ΔkdhAB mutant strain. (A) BALB/cByJ mice (n = 3) were challenged intranasally with LVS bacteria (10^3 CFU), ΔkdhAB bacteria (10^3 CFU), or PBS (control). BAL fluid was collected 24 h after infection, and the levels of TNF-α and IL-1β were measured by ELISA (left panel). The bacterial burden in lung was measured 24 h after infection (right panel). (B) Levels of TNF-α and IL-1β released by ex vivo BMMs after stimulation with LVS or ΔkdhAB bacteria (MOI of 10, 20, or 50). Culture supernatants were analyzed by ELISA 24 h after infection. (C) TNF-α and IL-1β gene expression in BMMs infected with LVS or ΔkdhAB bacteria (MOI of 10). Gene expression was measured by real-time PCR, normalized with RP II gene expression, and reported as relative expression compared with that in uninfected macrophages. All data are presented as mean plus SD (error bars) values (n = 3).
Characterization of kdhAB in Francisella tularensis

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Analysis of lipid A indicated that LVS and the assisted laser desorption ionization (21–24). Toward this end, the analysis of total LPS level and a complement, antibodies, and receptors of the innate immune system surface plays a vital role in preventing the binding of serum components and receptors to the bacterial cell membrane. In an alternative approach, we tested the filtered supernatants of LVS or ΔkdhAB bacteria for 2 h (MOI = 100). The IL-1β level in cell lysates was assessed by ELISA. Measurements were expressed as fold induction in infected versus uninfected cells (mean plus SD; n = 3). (B) HEK293 cells stably expressing TLR2 or TLR4/MD2 were transiently transfected with an NF-κB luciferase reporter plasmid and stimulated for 24 h with LVS or ΔkdhAB bacteria (MOI = 20) or with Pam3CSK (1 μg/ml) or E. coli LPS (100 ng/ml) as positive controls. Purified LPSs from LVS or ΔkdhAB bacteria (5 μg/ml) were tested in a mixture with soluble CD14 recombinant protein (5 μg/ml). The data are reported as relative luciferase units and represent fold induction of luciferase activity in the cell lysate compared with that in unstimulated cells (mean plus SD; n = 3).

**FIG 4** Bacteria of the *F. tularensis* LVS ΔkdhAB mutant strain activate NF-κB through the TLR2 signaling pathway. (A) Macrophage-like cell lines were stimulated with LVS or ΔkdhAB bacteria for 2 h (MOI = 100). The IL-1β level in cell lysates was assessed by ELISA. Measurements were expressed as fold induction in infected versus uninfected cells (mean plus SD; n = 3). (B) HEK293 cells stably expressing TLR2 or TLR4/MD2 were transiently transfected with an NF-κB luciferase reporter plasmid and stimulated for 24 h with LVS or ΔkdhAB bacteria (MOI = 20) or with Pam3CSK (1 μg/ml) or E. coli LPS (100 ng/ml) as positive controls. Purified LPSs from LVS or ΔkdhAB bacteria (5 μg/ml) were tested in a mixture with soluble CD14 recombinant protein (5 μg/ml). The data are reported as relative luciferase units and represent fold induction of luciferase activity in the cell lysate compared with that in unstimulated cells (mean plus SD; n = 3).

Strains (Fig. 5A) strongly supported the notion that the ΔkdhAB mutation does not compromise cell membrane integrity. In an alternative approach, we tested the filtered supernatants of LVS or ΔkdhAB cultures for their ability to activate BMMs in vitro. The culture supernatants were concentrated by Centricon filter devices and incubated with BMMs for 24 h. Macrophage activation was measured by the release of TNF-α and IL-6. While the addition of LPS or Pam3CSK (as positive controls) to the BMMs did result in significant activation of the cells, little activation was seen after the addition of LVS or ΔkdhAB mutant-conditioned media (see Fig. S3 in the supplemental material). The similarly low levels of TNF-α and IL-6 for both LVS and ΔkdhAB organisms suggested that there is no leakage of potential TLR2 ligands due to the ΔkdhAB mutation.

Unable to detect a leaky-membrane phenotype, we next investigated changes in bacterial LPS and cell-surface protein availability. It has been proposed that fully formed LPS on the bacterial surface plays a vital role in preventing the binding of serum complement, antibodies, and receptors of the innate immune system (21–24). Toward this end, the analysis of total LPS level and a characteristic LPS banding pattern showed no difference between LVS and the ΔkdhAB mutant (Fig. 6A). Additionally, matrix-assisted laser desorption ionization—time of flight (MALDI-TOF) analysis of lipid A indicated that LVS and the ΔkdhAB mutant have similar lipid A mass values (Fig. 6B). Although the ΔkdhAB mutation does not affect the total LPS level or the characteristic banding pattern of LPS, we thought it is possible that the additional Kdo—a negatively charged sugar—in the ΔkdhAB mutant alters the conformation of LPS molecules, thereby making other surface constituents more accessible to TLR2 receptors. To test this hypothesis, we evaluated the binding of FopA antibodies to the FopA surface protein in vitro. Live bacteria were incubated with mouse antibodies to FopA, and surface-bound antibodies were analyzed by Western blotting. Consistent with a previous study (24), fully formed LPS significantly reduced the binding of antibodies to the LVS bacterial surface, whereas ΔwbdA mutant LPS, which is devoid of O polysaccharide, did not (Fig. 5B). Strikingly, substantial binding of FopA antibodies to the ΔkdhAB mutant bacterial surface was found, and this result suggested that a TLR2 ligand present on the bacterial cell surface would be potentially exposed for detection by host immune-cell TLR2 receptors. The accessibility of surface proteins might well explain the enhanced TLR2 signaling activity caused by the ΔkdhAB mutation.

**Biological impact of TLR2 activation by the ΔkdhAB mutant.** We next addressed the biological importance of the TLR2-mediated response to ΔkdhAB bacteria in a mouse infection model. WT C57BL/6, TLR2−/−, and TLR4−/− mice were infected with various doses of the ΔkdhAB mutant via the intranasal route and monitored for survival. Most strikingly, at a dose of 10⁶ CFU, all WT and TLR4−/− mice survived, while no TLR2−/− mice survived (Table 1). The results suggest that TLR2-mediated responses play a significant role in the survival of mice infected with the *F. tularensis* ΔkdhAB mutant strain. Thus, the association of Kd-hAB Kdo hydrolase with enhanced TLR2-mediated inflammation points to a critical role for this enzyme complex in the virulence of LVS.

**Immunization with *F. tularensis* LVS ΔkdhAB mutant protects against the highly virulent *F. tularensis* Schu S4 strain.** Immunization with LVS is protective against the fully virulent *F. tularensis* type A strain Schu S4 in a mouse model of infection, and this protection involves TLR2-dependent innate immunity (25–27). However, even in low numbers, LVS retains significant virulence in mice. Our findings that the ΔkdhAB mutant is further attenuated and elicits enhanced TLR2 signaling led us to investigate the protective capacity of this mutant strain as a live cell vaccine. To this end, BALB/cByJ mice were immunized with three intranasal doses of LVS, the ΔkdhAB mutant, or (as a control) PBS. Four weeks after the last dose, the mice were challenged via the intranasal route with either LVS at a dose of 10⁶ CFU (10 x LD₅₀) or the Schu S4 strain at a dose of 10 CFU (10 x LD₅₀). All mice that had been immunized with LVS survived LVS challenge, whereas all control mice that had received PBS succumbed within 9 days (Fig. 7A). The ΔkdhAB mutant was also fully protective against LVS challenge (Fig. 7A). Furthermore, as expected, LVS-immunized mice survived the fully virulent Schu S4 challenge, while all PBS-treated control mice succumbed within 7 days (Fig. 7B). Strikingly, mice immunized with the ΔkdhAB mutant were well protected; 90% of these mice survived an otherwise lethal challenge with Schu S4. Histologic examination of the livers from infected mice revealed that ΔkdhAB mutant-immunized mice had much less tissue necrosis after challenge with Schu S4 (Fig. 7C) than did PBS-treated control mice. The protection conferred by the ΔkdhAB mutant was also reflected by lower bacterial loads in the livers, spleens, and lungs from mutant-immunized mice.
mice (Fig. 7D) than in those tissues from PBS-treated control mice. The Schu S4 bacterial loads in organs were similar in LVS- and ΔkdhAB mutant-immunized mice. Finally, the levels of Francisella-specific IgG antibody were slightly higher in the sera of ΔkdhAB mutant-immunized mice compared to LVS-immunized mice (Fig. 7E).

**DISCUSSION**

Evasion of innate immunity is a key virulence factor for a number of pathogens, including *F. tularensis* (6, 7). To avoid detection by pattern recognition receptors such as TLRs, *F. tularensis* modifies its lipid A through the action of enzymes such as 4′-phosphatase, galactosamine transferase, and mannosyl transferase (8, 10). A novel LPS modification was hypothesized in the lipid A through the action of enzymes such as 4′-phosphatase, galactosamine transferase, and mannosyl transferase (8, 10). A novel LPS modification was hypothesized in

![FIG 5 Mutation in kdhAB leads to enhanced accessibility of the bacterial surface. (A) Investigation of the leaky-membrane phenotype. Mid-logarithmic-phase cultures were spread onto CHAH plates, and a 10-μl volume of SDS (20, 10, and 5%) or EDTA (500, 250, and 125 mM) was spotted onto filter discs. Images were captured after 48 h of bacterial growth. Black arrows indicate zones of inhibition around 6-mm filter discs. (B) *F. tularensis* LVS and ΔkdhAB and ΔwbtA mutant strains were tested for binding of mouse anti-FopA antibodies to their surface FopA proteins in an antigen accessibility assay. Bacteria—along with surface-bound FopA antibodies—were lysed, and proteins were resolved by 5 to 20% SDS-PAGE. Bound FopA antibodies were detected as IgG heavy chain (HC) and IgG light chain (LC). Total FopA and bacterioferritin (Bfr) protein levels are shown as protein loading controls.

Kdo from its LPS (Fig. 1), was impaired in its ability to kill mice after intranasal challenge (Fig. 2). This attenuation was not due to a growth defect or to sensitivity to complement-mediated killing. In *H. pylori*, Kdo hydrolase inactivation has downstream effects on subsequent steps in LPS biosynthesis, reducing the efficiency of lipid A modification enzymes 4′-phosphatase and 3′-O-deacylase and the amount of fully extended O antigen (16, 17). Both 4′-phosphatase and O antigen are required for full virulence of *F. tularensis* in mice (10, 19, 29). However, Kdo hydrolase inactivation in *F. tularensis* LVS did not affect lipid A or O-antigen biosynthesis (Fig. 6). Therefore, virulence attenuation was not related to a defect in 4′-phosphatase activity or O-antigen assembly.

The avoidance of the TLR response by modifications in lipid A structure has been described in a number of pathogens, including members of the genera *Salmonella*, *Yersinia*, *Pseudomonas*, and *Francisella* (8, 10, 30–32). These modifications include lipid A deacylation, dephosphorylation, and glycosylation. We thus hypothesized that *F. tularensis* removes side chain Kdo from LPS core oligosaccharide to avoid the triggering of innate immunity. In agreement with this hypothesis, the ΔkdhAB mutant stimulated a stronger TLR2-mediated proinflammatory response in macrophage monolayers than the parental LVS did (Fig. 3 and 4). In *vivo*, ΔkdhAB bacteria induced a proinflammatory response in the lungs 24 h after intranasal challenge, while LVS bacteria did not (Fig. 3A, left panel). Attenuation of the virulence of ΔkdhAB bacteria may be related to this early innate immune response. Indeed, several studies have shown that eliciting an early inflammatory response in mice by priming the animals with an agonist of TLR3, TLR4, or TLR9 enhances survival to *F. tularensis* infection and decreases organ bacterial burdens (33–35).

Recent studies have indicated that Kdo residues contribute to proinflammatory responses to LPS (36–38). Therefore, the presence of a side chain Kdo in the ΔkdhAB mutant, with direct recognition of this modified LPS by the TLR2 signaling pathway, has emerged as a potential mechanism for the observed phenotype. However, our *in vitro* studies suggest that this is not the case, as purified LPSs from the ΔkdhAB mutant and LVS elicit similarly weak NF-κB activation (Fig. 4B). Another possible explanation is that the structural alteration of LPS exposes one or more TLR2 ligands present on the bacterial surface. The electrostatic charge of LPS is important for the folding of certain outer membrane proteins (39–42), and Kdo—as a negatively charged sugar—contributes to the overall charge of LPS. Thus, the presence of an additional Kdo residue in ΔkdhAB mutant LPS may alter the conformation of the outer membrane, exposing TLR2 ligands such as lipoproteins (43). In support of this idea, we showed that FopA surface protein is more accessible to antibodies in the ΔkdhAB mutant than in parental LVS (Fig. 5B).

LVS is an attenuated *F. tularensis* subsp. *holarctica* strain (type B) that was empirically derived in the former Soviet Union and developed as a live attenuated vaccine against the highly virulent *F. tularensis*. However, the LVS-based vaccine remains unlicensed in the United States and several other nations for several reasons, including the following: (i) an incomplete understanding of the genetic basis for its attenuation; (ii) untoward reactions; and (iii)
concerns about possible reversion back to the virulent form. The mechanisms of LVS-mediated protection against tularemia involve TLR2-dependent innate immunity (25–27). Therefore, an LVS mutant that exhibits attenuated virulence in mice and more strongly elicits activation of TLR2 signaling might prove to be a safer and more effective vaccine candidate than the original LVS. Here we show that the \( \Delta kdhAB \) LVS mutant may be such a vaccine candidate: its virulence is attenuated, its ability to activate TLR2 is enhanced, and its protective efficacy against \( F. \) tularensis type A strain Schu S4 is similar to that of LVS. Mutants unable to modify lipid A by carbohydrate incorporation were attenuated in mice, activated proinflammatory responses, and were protective against \( F. \) tularensis subsp. novicida (8). However, protection against the highly virulent \( F. \) tularensis type A strain Schu S4 has not been investigated. Few previously assessed mutants have been shown to induce protective immunity against type A strains (for a review, see reference 44). These mutants have had deletions in genes including \( \text{FTT1103, FTT0918, sodB, wzy, and ggt (45, 46).} \) We report a new mutation that is relevant for tularemia vaccine design.

Herein we demonstrate that the two-component Kdo hydrolase KdhAB is involved in the virulence of \( F. \) tularensis LVS. A \( \Delta kdhAB \) mutant is attenuated in mice, elicits a stronger proinflammatory cytokine response in a TLR2-dependent manner, and induces protective immunity against the highly virulent Schu S4 strain of \( F. \) tularensis. Our data suggest that a novel LPS modification—the removal of side chain Kdo—is important in the evasion of innate immunity by \( F. \) tularensis. Kdo hydrolase is also produced by two other pathogens, \( H. \) pylori and \( L. \) pneumophila (14, 15, 17, 18). It will be particularly interesting to study the role of this enzyme in the virulence of these organisms.

MATERIALS AND METHODS

Ethics statement. All work with animals was conducted according to the recommendations in the Guide for the Care and Use of Laboratory Animals (47). The protocol was approved by the Standing Committee on Animals of the Harvard Medical Area (protocol 04723).

Bacterial strains and plasmids. Francisella tularensis LVS (kindly provided by Karen Elkins, U.S. Food and Drug Administration, Bethesda, MD) was grown in cysteine heart agar supplemented with 1% hemoglobin (CHAH) for 72 h at 37°C and 5% CO\(_2\), in modified Muller-Hinton broth (Becton, Dickinson, Franklin Lakes, NJ) supplemented with ferric pyrophosphate and IsoVitaleX (Becton, Dickinson, Franklin Lakes, NJ), or in tryptic soy broth (Difco, Detroit, MI). Escherichia coli strain DH5\( \alpha \) was used for general cloning and was grown either in Luria-Bertani (LB) broth or on LB agar plates. When appropriate, ampicillin (100 \( \mu \)g/ml) and kanamycin (50 \( \mu \)g/ml) were added to broth medium, and kanamycin (10 \( \mu \)g/ml) was added to CHAH.

Construction of the \( F. \) tularensis LVS \( \Delta kdhAB \) deletion mutant. The \( \Delta kdhAB \) mutant was constructed by allelic exchange (48) by a procedure described previously (14). The whole \( \text{kdhA} \) gene was deleted. As the last
eight bases of kdhA overlap with kdhB, the first eight bases of kdhB were also deleted. The following primers were used: 5′-ACC CCCC GG CTC TGGTGT GATGAT TATATAT AT (Xmal site underlined) and 5′-TGAGTTTATGTTAGTTATAT ATTTTTTTTTTTAT (KpnI site underlined) to amplify the 1-kb upstream region; 5′-ACC CCCC GG CTC TGGTGT GATGAT TATATAT AT (Xmal site underlined) and 5′-TGAGTTTATGTTAGTTATAT ATTTTTTTTTTTAT (KpnI site underlined) to amplify the 1-kb downstream region.

For complementation studies, plasmid pkdhAB encoding kdhAB genes under a groEL promoter was constructed. In brief, a DNA sequence containing open reading frames of both kdhA and kdhB genes was amplified by PCR with primers 5′-TGAGTTTATGTTAGTTATAT ATTTTTTTTTTTAT (EcoRI site underlined) and 5′-TGAGTTTATGTTAGTTATAT ATTTTTTTTTTTAT (Xhol site underlined). After restriction endonuclease digestion, the kdhAB DNA fragment was ligated into the EcoRI and Xhol sites of plasmid pTH17 (49).

Bacterial DNA/RNA preparation. Plasmids were introduced into F. tularensis LVS by electroporation, and miniplasmids for E. coli DH5α and F. tularensis LVS were prepared with a QIAprep spin miniprep kit according to the manufacturer’s instructions (Qiagen, Venlo, Nether-
lands). Restriction enzymes and DNA-mediating enzymes were pur-
chased from New England Biolabs. DNA ligations and transformations of chemically competent E. coli were performed by standard protocols (50).

LPS purification. LPS was purified from F. tularensis WT and ΔkdhAB strains as described elsewhere (49). A modification of the hot phenol-
water method was used (51).

Mouse infection and immunization studies. Male BALB/cByJ, C57BL/6, TLR2−/−, and TLR4−/− specific-pathogen-free mice (6 to 8
weeks old; Jackson Laboratory, Bar Harbor, ME) were housed in an animal
facility at Harvard Medical School. All procedures conformed to pro-
tocols approved by the Institutional Animal Care and Use Committee.
Bacterial virulence was assessed by intranasal challenge of BALB/cByJ
mice (n = 6) with 10^8 CFU of F. tularensis LVS bacteria or with 10^8, 10^9, or
10^10 CFU of ΔkdhAB bacteria (inoculum volume, 50 μl per mouse). Mice
were lightly anesthetized with isoflurane (Baxter, Deerfield, IL). The
importance of the TLR2 signaling pathway in mouse survival after intranasal
challenge with ΔkdhAB mutant bacteria (10^6, 10^8, or 10^10 CFU) was
assessed in C57BL/6 WT, TLR2−/−, and TLR4−/− mice. In all these in-
stances, survival was monitored over a 26-day period unless otherwise
indicated.

For immunization studies, groups of BALB/cByJ mice (n = 10) were
immunized by the intranasal route with three doses of bacteria at 2-week
intervals; the doses were 10, 10^3, and 10^9 CFU for F. tularensis LVS and 10^9,
10^7, and 10^9 CFU for the ΔkdhAB mutant. A mock immunization group
that received PBS served as a control. Four weeks after the last dose, mice
were challenged intranasally with either 10^6 CFU of LVS (10^6 × 10^6) or
10^9 CFU of strain Schu S4 (10^6 × 10^6). Mice were challenged with the F.
tularensis Schu S4 strain at the animal biosafety level 3 (ABS3) core
facility at Harvard Medical School. Titters of serum antibody to LPS were
measured by ELISA as described previously (26).

Organ bacterial burden and histopathology. BALB/cByJ mice were
challenged intranasally with 10^9 CFU of F. tularensis LVS or ΔkdhAB bac-
teria. Six days after infection, mice were killed, blood was collected, and
tissues were weighed, hand mashed, and homogenized with a Stomacher
80-paddle action blender (Seward, Port Saint Lucie, FL). Serial 10-fold
dilutions were prepared with sterile 1× PBS supplemented with 2% fetal
bovine serum. A 10-μl volume of each dilution was plated onto CHAH
plates and incubated at 37°C in 5% CO2 until colonies became visible
(72 h). Data were expressed as CFU per gram of tissue or per milliliter of
blood.

Histological analysis was performed on the livers of F. tularensis Schu
S4-challenged mice that had been immunized with F. tularensis LVS or
ΔkdhAB mutant or had been given PBS as a control. Livers from each
group of mice were fixed in 10% formalin solution, embedded in paraffin,
and stained with hematoxylin and eosin after sectioning.

Bronchoalveolar lavage. BALB/cByJ mice were challenged intrana-
sally with 10^9 CFU of F. tularensis LVS or ΔkdhAB bacteria. After 24 h, the
mice were killed, the tracheae were exposed, and the lungs were lavaged
with 0.5 ml of PBS supplemented with 0.5 mM EDTA. TNF-α and IL-1β
were assayed with ELISA Duoset kits (R&D Systems, Minneapolis, MN).

BMM infection and measurements of cytokine production and gene
expression. Bone marrow was flushed with PBS from the femurs of
C57BL/6 mice (6 to 8 weeks old; Jackson Laboratory), spun down, resus-
pended in BMM growth medium (Dulbecco modified Eagle medium
[DMEM] containing d-glucose [4.5 mg/liter], l-glutamine [4 mM], and
sodium pyruvate [110 mg/liter] [Invitrogen, Carlsbad, CA] supple-
menced with 10% heat-inactivated fetal bovine serum [FBS] [Invitrogen]
and 30% cell line L929-conditioned DMEM with nonessential amino ac-
ids). The cells from each femur were plated in two deep petri dishes, and
the plates were incubated at 37°C in 5% CO2, in the presence of gentamicin
(50 μg/ml). After 3 days, BMM growth medium was added to petri dishes.
The cells were incubated for an additional 2 to 3 days.

For measurements of cytokine release, BMMs were seeded in 24-well
plates at a density of 5 × 10^8 cells/well, and the plates were incubated at
37°C in 5% CO2 overnight. The macrophages were then infected with mid-logarithmic-phase F. tularensis LVS or ΔkdhAB bacteria at an MOI of
10, 20, or 50 (ratio of bacteria to macrophages) for 24 h. TNF-α and IL-1β
in cell supernatants were measured with ELISA Duoset kits according to
the manufacturer’s recommendations (R&D Systems).

For measurements of gene expression, BMMs were seeded in 6-well
plates at a density of 2 × 10^6 cells/well, and the plates were incubated at
37°C in 5% CO2 overnight. The macrophages were then infected with
mid-log F. tularensis LVS or ΔkdhAB bacteria at an MOI of 10. At various
time points, cells were lysed in 0.4 ml TRizol (Invitrogen), and RNA was
isolated according to the manufacturer’s instructions. Reverse transcrip-
tion was performed with a qScript Flex cDNA synthesis kit (Quanta Bio-
sciences, Gaithersburg, MD) according to the manufacturer’s instruc-
tions. Real-time PCR was conducted with Perfecta SYBR green supermix
(Quanta Biosciences) in a Bio-Rad iCycle q system (45 cycles, with 1
cycle consisting of 15 s at 95°C, 45 s at 60°C, and 30 s at 72°C). Gene
expression was normalized to the expression of the RNA polymerase II
(RP II) gene, which is considered a stably expressed housekeeping gene
(52).

The following primer sets were used: for TNF-α, sense (5′-TATGG
CTCAGGTGTCACAATC-3′) and antisense (5′-CTCCTCTTTCGCAACT
CAGG-3′); for IL-1β, sense (5′-GCGGTCCTAAAGAAGAATC-3′) and
antisense (5′-TACCATGTTGGGAACCTCCTG-3′); and for IL-1β, sense
(5′-GCAACCGTCAATGATAT-3′) and antisense (5′-CTGCGCGTT
GACCCCATGAC-3′). Relative gene expression was calculated by the
ΔΔCt method (53) and reported as fold induction over the uninfected
control level (mean ± standard deviation [SD] values for samples from
three independent experiments).

Murine macrophase-like cell line infection. WT, Um93B−/−, Myd88−/−, TLR4−/−, and TLR2−/− murine macrophase-like cell lines,
kindly provided by Douglas T. Golenbock (University of Massachusetts
Medical School, Worcester, MA), were seeded in 24-well plates at a
density of 5 × 10^5 cells/well, and the plates were incubated overnight at
37°C in 5% CO2. The macrophages were then infected with mid-log F. tulare-
ensis LVS or ΔkdhAB bacteria at an MOI of 100. After 2 h, cells were
washed three times with Dulbecco’s PBS and lysed in 60 μl of lysis buffer
(Kit BFI14100; R&D Systems). IL-1β in lysates was measured with an
ELISA Duoset kit (R&D Systems).

NF-kB reporter luciferase assay. Stable HEK293 cell lines expressing
TLR2 receptor were kindly provided by Douglas T. Golenbock. HEK
TLR2 and HEK-TLR4/MD2 cells were seeded into 12-well tissue culture
plates at a density of 3 × 10^5 cells/well. The following day, cells were
transiently transfected with luciferase reporter genes; Genejuice (EMD
Chemicals, Darmstadt, Germany) was used per the manufacturer’s rec-
ommendations. To assess NF-kB activation, an NF-kB luciferase reporter
gene (consisting of an artificial promoter NF-kB site driving the firefly
luciferase gene) was cotransfected with a constitutively active Renilla
luciferase reporter gene, phRL-TK (Promega, Fitchburg, WI); phRL-TK
was used to normalize transfection efficiency. The following day, the cells
were stimulated as indicated in Results. During stimulation with LPSs
from F. tularensis LVS or ΔkdhAB bacteria, the mixture contained soluble
CD14 recombinant protein (5 μg/ml) (ProSci Inc., Poway, CA). After 24 h
of stimulation, the cells were lysed in passive lysis buffer (Promega),
and reporter gene activity was measured with a plate reader luminometer
(Victor2; PerkinElmer Life Sciences) and the Dual-Luciferase reporter
assay system (Promega).

Surface antigen-binding assay. Evaluation of FopA antibody binding
to the bacterial surface was performed as described previously (24), with
some modifications. In brief, cultures of WT F. tularensis LVS, ΔkdhAB,
and ΔwbtB bacteria were grown overnight in brain heart infusion (BHI)
broth. Cultures were diluted in fresh BHI medium to an optical density
at 620 nm (OD620) of 0.07, and bacteria were further grown to an OD620
of 0.5. After the bacteria were harvested, 6 × 10^8 bacteria from each strain
(in a 50-μl volume) were mixed with monoclonal FopA antibodies (final
concentration, 0.1 mg/ml) (54) in PBS containing 0.1% glucose and 0.5%
bovine serum albumin (BSA). After incubation for 1 h at 37°C, bacteria

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were washed three times with PBS containing 0.1% glucose. After the final wash, pellets were resuspended in Laemmli sample buffer (Bio-Rad, Hercules, CA). Protein samples were resolved on 5 to 20% Protean TGX gels (Bio-Rad) and transferred to nitrocellulose membranes. Alkaline phosphatase (AP)-conjugated rabbit anti-mouse antibody (Abcam, Cambridge, MA) was used to detect heavy- and light-chain subunits of bound FopA antibodies.

**LPS analysis by SDS-PAGE and MALDI-TOF mass spectrometry.** Outer membranes from *F. tularensis* LVS and ΔkdhAB bacteria were isolated, resolved by Tricine SDS-PAGE, and stained with zinc as previously described (14). Lipid A from LVS and ΔkdhAB bacteria was isolated and analyzed by negative-ion matrix-assisted laser desorption ionization—time of flight (MALDI-TOF) mass spectrometry (14). Data analysis. Data are expressed as mean ± standard deviation (SD) values. Unpaired Student’s *t* test was used for statistical analysis. A *P* value of <0.05 was considered significant.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org /lookup/suppl/doi:10.1128/mBio.00638-12/-/DCSupplemental.

Figure S1, EPS file, 0.8 MB.

Figure S2, JPG file, 0.5 MB.

Figure S3, JPG file, 0.5 MB.

Table S1, DOCX file, 0.1 MB.

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