Published in final edited form as:

Microb Pathog. 2007 ; 42(5-6): 204–214. doi:10.1016/j.micpath.2007.02.001.

Modulation of virulence factors in *Francisella tularensis* determines human macrophage responses

Paul E. Carlson Jr¹, James A. Carroll¹, Dawn M. O'Dee¹, and Gerard J. Nau^{1,2,*}

¹ Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, 15261, USA

² Department of Medicine - Division of Infectious Diseases, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, 15261, USA

Abstract

Francisella tularensis, the causative agent of tularemia and Category A biodefense agent, is known to replicate within host macrophages, though the pathogenesis of this organism is incompletely understood. We have isolated a variant of *F. tularensis* Live Vaccine Strain (LVS) based on colony morphology and its effect on macrophages. Human monocyte-derived macrophages produced more tumor necrosis factor α (TNF α), interleukin (IL)-1 β , IL-6, and IL-12 p40 following exposure to the variant, designated the activating variant (ACV). The immunoreactivity of the lipopolysaccharide (LPS) from both LVS and ACV was comparable to the previously described blue variant and was distinct from the gray variant of LVS. We found, however, the soluble protein fractions of LVS and ACV differed. Further investigation using two-dimensional gel electrophoresis demonstrated higher levels of several proteins in the parental LVS isolate. The differentially-expressed proteins featured several associated with virulence in *F. tularensis* and other pathogens, including intracellular growth locus C (IglC), a σ^{54} modulation protein family member (YhbH), and aconitase. ACV reverted to the LVS phenotype, indicated by low cytokine induction and high IglC expression, after growth in a chemically-defined media. These data provide evidence that the levels of virulence factors in *F. tularensis* are modulated based on culture conditions and that this modulation impacts host responses. This work provides a basis for investigation of *Francisella* virulence factor regulation and the identification of additional factors, co-regulated with IglC, that affect macrophage responses.

Keywords

Francisella; macrophage; cytokine; virulence factors

Introduction

Francisella tularensis, the causative agent of tularemia, is a facultative intracellular bacterium, known to replicate in macrophages [1]. *F. tularensis* subspecies *tularensis* is highly infectious, with intradermal or inhalation routes of infections requiring only about 10 organisms to cause serious disease [1]. If untreated, death rates from pulmonary tularemia can be as high as 60%

*Corresponding Author: Gerard J. Nau, Department of Molecular Genetics and Biochemistry, E1256 BSTWR, 200 Lothrop St., Pittsburgh, PA 15261, Tel: 412-383-9986, Fax: 412-624-1401, Email: E-mail: gjnau@pitt.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

[2]. These factors have led to concerns over the potential use of *F. tularensis* as a biological weapon, leading to its classification as a Category A biodefense agent [2]. Although there is a vaccine for this disease, the live vaccine strain (LVS) was not licensed for use because of both its inability to induce complete protection and its unknown mechanism of attenuation [3]. LVS, which was derived from *F. tularensis* subspecies *holarctica*, exhibits minimal virulence in humans, but has been used extensively to study tularemia due to its virulence in murine models [4].

Macrophages are important components of the innate immune system, functioning to engulf and kill microorganisms. Some bacteria, including *Francisella*, survive and replicate within these cells [5], emphasizing the value of examining the interaction between macrophages and *Francisella*. It is also important to understand *Francisella* in the context of human infection. Though these organisms have been intensely studied in the murine model [4], human responses to these pathogens is less well studied [6,7]. Similarly, investigation into the cytokine response these organisms elicit from human macrophages has been limited [8].

Several important virulence determinants have been identified in *F. tularensis*. LPS [1] and capsule [9] contribute to virulence because the LPS induces only low levels of proinflammatory cytokines, IL-1 β and TNF α , while the capsule improves resistance to complement. In addition, specific proteins are required for intramacrophage growth by *F. novicida* [10] and *F. tularensis* LVS [11], including the *iglABCD* operon [12–18] and the *pdpA* and *pdpD* genes [11] contained within the *F. tularensis* pathogenicity island [11]. Nevertheless, the functions of these proteins and the overall pathogenesis of *F. tularensis* remain incompletely understood.

In conducting studies of host cell responses to *F. tularensis* LVS, we have isolated a variant that differed from the parental LVS strain in colony morphology and in the ability to elicit cytokines from human macrophages. This variant appears distinct from previously identified variants [19]. Our closely related *F. tularensis* isolates were used to identify putative virulence factors that may influence macrophage responsiveness. Moreover, we show that culture conditions modulate the level of virulence factor expression in this organism. Comparing the LVS and ACV isolates will facilitate identification of putative *Francisella* virulence factors that are co-regulated with IgIC.

Results

Isolation of a novel LVS variant

While examining macrophage responses to *F. tularensis* LVS, we identified a variation of LVS colony morphology. The variant emerged from LVS cultures grown to an OD₆₀₀ > 0.8 in Mueller-Hinton (MH) broth. Colonies of LVS had a bright white appearance, while the variant colonies exhibited a more dull color on 5% sheep blood tryptic soy agar plates. Once generated, the phenotypes of these isolates were stable on sub-culturing in MH broth (LVS parent isolate was stable to OD₆₀₀ < 0.25) or chocolate II agar plates. The LVS variant reproducibly arose from high density cultures and individual colonies derived from those cultures had a similar appearance on tryptic soy agar plates with 5% sheep blood. Re-culturing the variant at low densities in MH broth failed to revert the variant to the original LVS appearance (data not shown).

Human macrophage response to *Francisella* isolates

Since previous reports have shown blue and gray LVS variants induce different responses from rat macrophages [19], we tested our LVS and variant for their ability to activate macrophages. LVS and our colony variant were added to cultures of human monocyte-derived macrophages and supernatants were harvested after 24 hours. We found that macrophages produced higher

levels of TNF α , IL-1 β , IL-6, and IL-12 p40 in response to the variant compared to the parental LVS (Fig. 1). In fact, there was no detectable IL-1 β or IL-12 p40 induced by the parental LVS (Fig. 1B, D). Because the variant induced higher levels of proinflammatory cytokines, we termed this an **activating variant (ACV)** of LVS.

Since some published reports have used higher doses of organisms during *in vitro* infection experiments [20,21], it was possible that the low macrophage response to LVS observed in our experiments was due to an inadequate bacterial inoculum. To test this possibility, we examined the macrophage response to different numbers of bacteria. Macrophages were co-cultured for 24 hours with a range of MOIs. Even at an MOI of 100, LVS elicited substantially less TNF α than ACV (Fig. 2A). Forty to sixty percent of macrophages exposed to MOIs in this range for four hours were associated with bacteria by fluorescence microscopy. Therefore, LVS failed to induce cytokines even though there was a demonstrable interaction between the bacteria and the macrophages.

It was possible that ACV expressed ligands for Toll-like receptors (TLR), while LVS did not, or that such ligands were hidden from macrophage recognition in LVS. To address these possibilities, we compared whole cell lysates from LVS and ACV for their ability to activate human macrophages. Lysates from both isolates elicited comparable dose-response curves of TNF α production from human macrophages (Fig. 2B). Comparable levels of cytokine secretion were also observed when macrophages were exposed to either isolate killed by freeze-thaw (data not shown). These data demonstrate that components of LVS are capable of activating macrophages, suggesting these components were masked in live bacteria or that live LVS actively inhibited host responses. In contrast, ACV appears to have lost this ability to mask its stimulatory components or inhibit host responses.

Wild type *F. tularensis* LVS inhibits normal TLR4 signaling [22] and we speculated that ACV lost this property, providing an explanation for the ability of ACV to induce higher levels of cytokines from macrophages (Fig. 1). To test this, macrophages were cultured for 4 hours with LVS or ACV such that 70% of the macrophages contained bacteria on immunofluorescence staining (data not shown). The macrophage cultures were then washed after the 4 hour incubation to remove extracellular bacteria, and *E. coli* LPS was added to stimulate TNF α production. As we have seen previously (Fig. 1A), LVS elicited low levels of TNF α while ACV induced high levels of the cytokine (Fig. 3). As expected, LPS alone also induced high levels of TNF α (Fig. 3). Macrophages pre-incubated with ACV and then cultured with LPS produced the highest levels of TNF α . In contrast, pre-incubation with LVS significantly inhibited TNF α induction by LPS (Fig. 3). These results suggested inhibition of TLR signaling was the mechanism for low cytokine responses to LVS compared to ACV.

F. tularensis has been shown to induce host cell death [20,21], raising the possibility that LVS caused rapid macrophage death, thereby preventing cytokine production by the host cells. We assessed host cell viability after exposure to the LVS isolates by two strategies. First, we measured release of lactate dehydrogenase (LDH) from the cytoplasm of macrophages after incubation with LVS or ACV for 24 hours, as has been done previously [8]. No differences in LDH release were observed between macrophages cultured with LVS and ACV ($p = 0.7$ by t-test) (Table 1). The second strategy used fluorescent stains that distinguish between live and dead cells (Live/Dead staining kit, Invitrogen). The results were similar to those obtained measuring LDH release in that no significant differences in macrophage viability were seen following exposure to LVS or ACV ($p = 0.88$ by t-test) (Table 1). LVS and ACV at high MOI, such as that used in the TLR inhibition experiments (Fig. 3), did not result in appreciable differences in macrophage viability (data not shown). These data suggested that macrophage death was unlikely to account for the differences in cytokine production following exposure to the LVS isolates.

Molecular characterization of LVS isolates

We next set out to determine the molecular basis for the differential macrophage response to the LVS isolates. A blue to gray colony morphology variation has been described in LVS, where the gray variants are more proficient at activating host macrophages [19]. These blue/gray variants can be distinguished by immunoreactivity of their LPS. Blue strains react only with antibodies against *F. tularensis* LPS. Gray strains react with antibodies recognizing both *F. tularensis* LPS and *F. novicida* LPS [19]. Following proteinase K treatment of whole cell lysates from LVS and ACV, we compared the antibody reactivity of the remaining material to samples isolated from *F. novicida*. An immunoblot of LPS from both LVS and ACV showed that both isolates reacted with the anti-*F. tularensis* LPS antibody and neither bound the anti-*F. novicida* LPS antibody (Fig. 4). In contrast, the LPS isolated from *F. novicida* reacted strongly with the anti-*F. novicida* LPS antibody. Using the molecular definition put forth by Cowley et al., our results indicate that both the LVS and ACV isolates exhibit the LVS blue phenotype because both reacted only with the anti-*F. tularensis* LPS antibody.

Since the immunoreactivity of the LPS was similar in the two isolates, we investigated the protein content of LVS and ACV to determine why they elicited different macrophage cytokine responses. As a first test, whole cell lysates, membrane proteins, or soluble proteins from each isolate were separated on single dimension acrylamide gels and silver stained to characterize bacterial proteins (Fig. 5). The protein content in whole cell lysates and membrane fractions of LVS and ACV were indistinguishable using this technique. The soluble fractions of LVS and ACV, however, demonstrated a number of differences observed by this method (Fig. 5, arrows).

We pursued the differences in the soluble protein fractions of LVS and ACV using two-dimensional gel electrophoresis and silver staining. Using this approach, we found the majority of proteins to be similar in abundance between LVS and ACV when equal amounts of protein were loaded on the gel. However, six proteins were identified that were qualitatively greater in LVS than in ACV (Fig. 6). The spots containing these proteins were isolated and identified by MALDI-TOF (Table 2). One of the proteins identified was intracellular growth locus C (IglC), a known *F. tularensis* virulence factor [13–18]. Also included were aconitase, which is part of the TCA cycle and has been associated with *Staphylococcus aureus* virulence [23], and a homolog of a σ^{54} modulation protein (YhbH), which has known functions in sporulation [24], biofilm formation [25], and quorum sensing [26]. Other identified proteins included fumarate hydratase, a protein involved in the TCA cycle, and a heat shock protein (Hsp20). Proteins that appeared more abundant in ACV (Fig. 5) were not identified on the two-dimensional gels (Fig. 6). This was most likely related to differences in protein solubilization between the techniques; SDS was used in one-dimensional SDS-PAGE. In addition, a narrower portion of the pH range (pH 4 to 8.5) was resolved in the isoelectric focusing step of the two-dimensional gel protocol than the total proteins contained in one-dimensional gels. Nevertheless, qualitative differences in protein content were observed using the two-dimensional electrophoresis technique.

We next sought to confirm the results obtained by two-dimensional gels with another technique. To do this, we measured IglC levels by immunoblot to quantify the difference between LVS and ACV whole cell lysates. IglC levels were 15-fold higher in LVS than in ACV (Fig. 7), confirming the qualitative results observed with silver stain. We have previously observed that Hsp70 levels of LVS and ACV were comparable on two-dimensional gels and immunoblots derived from two-dimensional gels, and used this to confirm protein loads. The membrane was re-probed with anti-Hsp70 polyclonal antibody showing similar levels of Hsp70 in the 1 μ g of whole cell lysates that were loaded in each lane (1.2-fold difference) (Fig. 7).

We next assayed the expression of several genes to determine if RNA levels correlated with the protein levels observed on the two-dimensional gels (Fig. 6). We selected *acnA*, *iglC*, and *yhbH* for this test as each has been associated with bacterial virulence [14,17,23–26]. The abundance of these genes' transcripts was measured by quantitative PCR using 16S rRNA as an internal reference. Surprisingly, the expression of the *iglC*, *acnA*, and *yhbH* differed less than two-fold between LVS and ACV (data not shown). The presence of transcripts for these genes argues against the hypothesis that genomic deletions in ACV explain loss of IgIC, AcnA, and YhbH. Rather, the gene expression data suggest the amounts of these three proteins were controlled at a step after transcription.

Reversion of ACV to the LVS phenotype

We next sought to determine if ACV could revert back to the LVS phenotype. To investigate this, we tested different culture conditions and media, including a chemically defined media (CDM) originally described by Chamberlain [27]. For these experiments, LVS and ACV were cultivated in CDM or MH. The procedure for generating these experimental inocula is illustrated in figure 8A. As previously observed (Fig. 1), growing LVS to high density ($OD_{600} \geq 1.0$) in MH broth elicited the ACV phenotype and high levels of TNF α production from macrophages ("LVS MH", Fig. 8B). When ACV was used to inoculate MH broth, high levels of cytokine were observed again ("ACV MH", Fig. 8B). When LVS was grown to a high density in CDM ($OD_{600} \geq 1.0$), it retained its phenotype and induced little TNF α ("LVS CDM", Fig. 8B). More importantly, ACV grown in CDM behaved like LVS eliciting little TNF α ("ACV CDM", Fig. 8B). These results show that ACV reverts to LVS following cultivation in CDM.

We then tested whether the ACV reversion correlated with a change in IgIC after culture in the CDM. For this, we utilized the same bacterial cultures used to infect macrophages (Fig. 8B). Growth to a high density in MH resulted in low levels of IgIC, irrespective of the source of the initial inoculum ("LVS MH", "ACV MH"; Fig. 8C). This result is similar to our previous data showing low IgIC expression in ACV (Fig. 6, 7). In contrast, cultures grown in CDM had substantially higher levels of IgIC, once again irrespective of which strain was used to inoculate the culture ("LVS CDM", "ACV CDM"; Fig. 8C). These data indicate an ability of *F. tularensis* to modulate expression of its virulence factors based on its environment.

Discussion

Although *F. tularensis* can cause a life-threatening disease in humans, current knowledge of the host-pathogen interaction is incomplete, particularly in human cells. In the course of our studies, we have isolated a variant of *F. tularensis* LVS based on colony morphology. Comparing the response of human macrophages to both the parent isolate (LVS) and the variant (ACV), we found they induced different amounts of cytokines. ACV consistently induced higher amounts of each proinflammatory cytokine tested. Both of our isolates are classified as "blue" based on LPS immunoreactivity [19], so we sought other explanations for the differences in macrophage activation. By comparing the proteomes of these two closely related isolates, we found differences in protein levels for the well-characterized *F. tularensis* virulence factor IgIC, as well as proteins associated with virulence in other organisms. These differences likely contribute to the macrophage responses we observed (Fig. 1).

Our parent isolate exhibits a phenotype comparable to other published reports, while ACV appears unique. For example, Bosio and Dow showed that LVS does not induce detectable levels of TNF α from murine pulmonary macrophages or dendritic cells [28]. Our results are also consistent with other observations using human macrophages, where LVS induces significantly less TNF α than that elicited by *E. coli* or *E. coli* LPS [8]. ACV failed to recapitulate these typical LVS phenotypes. ACV induced high levels of TNF α and other pro-inflammatory

cytokines. The fact that our isolates were stable on sub-culturing on both chocolate agar and MH broth (grown to low density, $OD_{600} < 0.2$) provided a useful system to contrast the protein expression profiles of LVS and ACV.

Examination of soluble protein fractions by two-dimensional gel analysis permitted us to identify six protein spots that were expressed at higher levels in LVS (Fig. 6). Previous *Francisella* proteomics studies have catalogued LVS protein profiles [29,30], immune reactive proteins [31], or stress response proteins [32]. A proteomic comparison of three *Francisella* strains has been performed, identifying many protein differences [33]. However, most of these were mass and charge variants of proteins present in all three strains [33]. The relatedness of our isolates facilitated the identification of protein differences beyond size and charge variations. Importantly, we were able to correlate altered protein content (Fig. 6) with physiologic responses by host cells (Fig. 1, 8).

The differences between LVS and ACV proteomes included proteins with roles in bacterial virulence. The most notable of these is intracellular growth locus C (IglC), a virulence factor known to play a role in *F. tularensis* and *F. novicida* intramacrophage survival [10,11,13,14]. Increased expression of IglC has also been demonstrated following growth in macrophages and exposure to hydrogen peroxide [12], suggesting a role for this protein in the stress response of the bacteria. Strains deficient in IglC are unable to grow within macrophages or escape from phagosomes [11,14,17]. LVS also inhibits TLR signaling in macrophages and IglC mutants do not manifest this phenotype [22]. ACV exhibits a phenotype similar to an IglC mutant, and is unable to inhibit TLR signaling (Fig. 3). It is likely that the lower abundance of IglC in ACV (Fig. 6) contributes to the heightened macrophage response induced by this strain.

Other proteins we identified have been associated with virulence in other systems. Aconitase, a TCA cycle protein that catalyzes the conversion of citrate to isocitrate, has been implicated in *S. aureus* virulence. Aconitase gene deletions in *S. aureus* reduce virulence factor expression and slow onset of subcutaneous infections in mice [23]. In *Pseudomonas aeruginosa*, aconitase contributes to synthesis of exotoxin A [34]. Although *F. tularensis* aconitase protein levels are lower in mouse spleen four days after inoculation than in broth culture [35], the role of this enzyme in other stages of *Francisella* infection has not been investigated.

We also found higher protein levels of YhbH, a member of the σ^{54} modulation protein family, in LVS. YhbH has been implicated in virulence of *Bacillus cereus* for its roles in sporulation [24] and biofilm formation [25]. In *E. coli*, YhbH is known to play a role in quorum sensing [26] and stabilizing ribosomes [36]. Biofilms and quorum sensing have important roles in virulence in several microbiological systems [37,38], yet these properties have not yet been described in *F. tularensis*. The ability to stabilize ribosomes could lead to increased translation of virulence factors, like IglC, or other proteins without necessarily increasing transcription. Improved expression of virulence factors and ability to translate proteins could enhance *F. tularensis* survival in hostile conditions.

Although multiple differences were observed between LVS and ACV at the protein level, mRNA levels were similar for all three of the genes tested. This indicates that loss of genetic material is not the mechanism of the variation observed and suggests post-transcriptional events, such as differential protein stability and selective protein degradation, may regulate the expression of proteins like IglC. Importantly, regulation of translation has been implicated in the control of genes associated with virulence in several other organisms such as *Pseudomonas aeruginosa*, *Listeria monocytogenes*, and *Burkholderia cenocepacia* [39–42].

Our results provide a molecular explanation to other publications regarding LVS, virulence, and host responses. CDM is known to enhance the virulence of LVS vaccine stocks [43]. Cherwonogradzky and colleagues showed growth of LVS in CDM increased capsule

production and virulence in mice by approximately 1000-fold [43]. Our data suggest levels of IgIC, and possibly other virulence determinants, increased by growth in CDM contribute to these observations. Loegering *et al.* recently demonstrated LVS grown in a murine macrophage-like cell line, RAW 264.7, induce lower amounts of proinflammatory cytokines than bacteria grown broth cultures [44]. Our results suggest the LVS grown in MH for their studies had shifted to the ACV variant, inducing cytokines because of a low level of IgIC. Passage of their bacteria through macrophages reconstituted the LVS phenotype with low cytokine induction, most likely because growth of LVS in macrophages increases IgIC expression [12].

Francisella response to environmental cues is now becoming an area interest and our results are among the first to demonstrate modulation, up and down, of *F. tularensis* virulence factor expression. Recently, Deng *et al* have shown that the expression of pathogenicity island genes are regulated by iron [45]. We have shown ACV reversion to LVS was observed following overnight growth of bacteria in CDM (Fig. 8). This phenotype switch was associated with reconstitution of IgIC levels. Our results differ from that of Deng *et al* because *iglC* expression levels appear similar in our system, implicating a post-transcriptional mechanism of regulation. The signal(s) that increase virulence factors in LVS and down-regulate macrophage responses and effects on virulence *in vivo* will be the focus of future studies.

We have shown the two isolates of *F. tularensis* LVS induce different amounts of proinflammatory cytokines from human macrophages, with the ACV inducing higher levels of all cytokines tested. Proteomic analysis of these isolates identified both known (IgIC) and candidate (AcnA, YhbH) *F. tularensis* virulence factors. The ability of ACV to revert to an LVS phenotype when grown in CDM provides evidence of virulence factor modulation based on environment, though the exact nature of the signals/receptors leading to these changes remains to be determined. Further study of the RNA and protein differences between LVS and ACV creates a unique opportunity to discover candidate *Francisella* virulence factors whose expression correlate with known virulence factors like IgIC and to investigate virulence factor regulation, ultimately increasing our understanding of *F. tularensis* pathogenesis.

Methods

Francisella strains and cultivation

F. tularensis LVS (ATCC #29684) and *F. novicida* (U112) were kindly provided by Dr. Karen Elkins (U.S. Food and Drug Administration). Fresh Mueller-Hinton (MH) broth supplemented with 0.1% glucose, 0.025% ferric pyrophosphate (Sigma), and Isovitalex was used to grow stocks of LVS. Bacteria for experiments, as well as further stocks, were grown from this initial passage. Low density broth cultures were pre-incubated at 37°C with 5% CO₂, then grown at 37°C with agitation until the cultures reached OD₆₀₀ ≤ 0.2, approximately 8–10 hours. Bacteria were then centrifuged and either used fresh, or resuspended in PBS + 20% glycerol and stored at –80°C until needed for infection. Infection experiments were performed using either frozen stocks, in which viable CFU had been determined by plating, or actively growing cultures. Actively growing cultures were diluted to an OD₆₀₀ associated with a narrow range of viable bacteria (LVS OD₆₀₀ = 0.075, approximately 3.0 × 10⁸ cfu/ml; ACV OD₆₀₀ = 0.075, approximately 2.0 × 10⁸ cfu/ml). Actual CFU of actively growing cultures were measured by plating serial dilutions on chocolate II agar plates at the start of the experiment. Similar results were obtained using either frozen stocks or overnight cultures of LVS. The activating variant (ACV) was isolated from cultures grown in MH broth to higher densities (OD₆₀₀ = 0.8–1.5). For routine experiments, ACV cultures were permitted to grow to OD₆₀₀ ≥ 1.0. To induce reversion of ACV to LVS, bacteria were cultured in a chemically defined media (CDM) described by Chamberlain in 1965 [27] overnight, to an OD₆₀₀ ≥ 1.0. For RNA and protein isolations, *F. novicida* and LVS isolates were grown on chocolate II agar at 37°C and 5%

CO₂ for one or three days, respectively, and harvested as indicated below. All bacterial growth media and supplements used in these experiments were purchased from BD Biosciences, unless otherwise stated.

Macrophage culture and infection

Human macrophages were differentiated from monocytes by *in vitro* culture. Elutriated human monocytes (> 95% purity) were purchased from Advanced Biotechnologies Inc. and cultured at a density of approximately 2.5×10^7 in 60mm culture dishes for seven days at 37°C with 5% CO₂ in 9ml of DMEM (Invitrogen) containing 20% FCS (Invitrogen), and 10% human serum (Gemini Biosciences). Some experiments were performed using monocytes isolated from human buffy coats of blood donations (Central Blood Bank, Pittsburgh). These cells were purified using Ficoll gradients (Amersham Biosciences) to isolate PBMCs, Optiprep gradients (Axis-Shield) to enrich for monocytes, and negative selection magnetic column separation (Miltenyi Inc.) or panning on plastic to further purify monocytes (final purity > 95% based on microscopy). These cells were then cultured similarly to the elutriated monocytes. On day seven, macrophages were removed from the culture dish using a lidocaine/EDTA solution (5 mM EDTA and 4 mg/ml lidocaine in PBS pH 7.2). Cells were washed and resuspended in DMEM containing 1% human serum prior to plating onto Primaria 96-well plates (BD-Falcon) at a density of 5.0×10^4 – 1.0×10^5 cells per well. Unless otherwise indicated, macrophages were exposed to bacteria at a multiplicity of infection (MOI) of approximately 10 for 24 hours before supernatants were collected. For TLR inhibition studies, macrophages were incubated with either LVS or ACV for four hours at an MOI of 500. The high MOI was used to increase the percent of macrophages (> 70%) infected following the short incubation. Macrophages were incubated with Hanks Balanced Salt Solution (HBSS) (Gibco) containing gentamicin (20 µg/ml) for 15 minutes to kill extracellular bacteria then washed three times with warm HBSS. *E. coli* LPS (1µg/ml) was then added to the macrophages for 20 hours before collection of supernatants. All use of human-derived cells was approved by the University of Pittsburgh Institutional Review Board.

ELISA analysis

Macrophage supernatants were harvested 24 hours after introduction of bacteria and were tested by ELISA analysis to measure cytokine levels. TNFα was measured using a matched antibody pair (R&D Systems) and IL-1β, IL-6, IL-12 p40 were measured using DuoSets (R&D Systems). Following the addition of TMB substrate solution (Dako) and measurement of optical density using a Molecular Dynamics M2 plate reader, cytokine levels were calculated from a standard curve. The limits of detection for the ELISAs were: TNFα – 15pg/ml, IL-1β – 15pg/ml, IL-6 – 15pg/ml, and IL-12 p40 – 10pg/ml.

Macrophage viability

Macrophage viability was examined following a 24 hour incubation with either media only or bacteria at an MOI of 10. At the end of the incubation, supernatants were harvested and tested for the presence of lactate dehydrogenase (LDH) using the Cytotoxicity Detection Kit^{PLUS} (Roche) according to manufacturer's protocol. Percent lysis was calculated by comparing samples to maximal LDH release from macrophages treated with the lysis buffer provided in the kit. Data are presented as % maximal LDH release where % max LDH = (sample LDH – background)/(maximum LDH – background) *100. Following the removal of supernatants from the macrophage cultures, the cells were tested immediately for staining with Invitrogen's Live/Dead staining kit according to manufacturer's protocol. SYTO9 (live) and propidium iodide (dead) staining was measured using a Molecular Dynamics M2 plate reader at the following excitation and emission wavelengths: SYTO9 480/500nm, propidium iodide 490/635nm.

Protein Electrophoresis and Immunoblotting

F. tularensis LVS, *F. tularensis* LVS - ACV, and *F. novicida* were harvested from chocolate II agar plates, suspended in 3ml of PBS, and lysed by French press using two passes at 18,000 lb/in². These whole cell lysates were then fractionated into membrane-associated and soluble proteins by ultracentrifugation at 314,000 x g for one hour as described [46]. For single dimension gels, 10µg of total protein was solubilized in 2x Lamelli buffer and loaded in each well. Proteins were separated through a 4% acrylamide stacking and 12.5% acrylamide resolving gel at 30mA prior to staining with Silver Stain Plus (Bio-Rad). Protein concentrations in whole cell lysates and soluble fractions were determined by D_C Protein assay (Bio-Rad), while membrane protein concentrations were determined by modified Lowry using BSA as a standard [47].

For LPS western blots, cell lysates from the two isolates (LVS and ACV) were treated with excess proteinase K (1.0 mg/ml) for 30 minutes at 37°C before being separated on a 4–12% Bis-Tris gradient gel (Invitrogen). Separated material was transferred to PVDF membrane using XCell II™ Blot Modules (Invitrogen), according to manufacturer's protocol. Following transfer and blocking with 5% (wt/vol) non-fat dry milk in TBST, membranes were probed with either a mouse anti-*F. tularensis* LPS monoclonal antibody or a mouse anti-*F. novicida* (#8.2) monoclonal antibody (both from Immuno-Precise Antibodies Ltd.). Anti-mouse IgG antibody conjugated to Alexa Fluor 488 (Invitrogen) was used for detection and fluorescence was visualized on a Typhoon 9410 (Amersham Biosciences).

For two-dimensional gel electrophoreses, soluble protein fractions were isolated as described above and then precipitated by 80% acetone (v/v) and 10% TCA (v/v) and pelleted by centrifugation (16,000 x g, 20 min, 4°C). Protein pellets were suspended in C₄TT₃₋₁₀ solubilizing solution [48] and incubated overnight at 23°C with gentle agitation. Soluble material was clarified by ultracentrifugation (314,000 x g, 1 hr, 23°C), and 200µg of protein was loaded onto 13 cm, pH 3 to 10 linear IPG strips and focused in the first dimension for 82,000 Vh using the IPGphor system (Amersham Biosciences) (running conditions: 500V for 1hour, 1500V for 1 hour, 8000V for 80,000 Vh). Focused strips were stored at -80°C until separated in the second dimension by SDS-PAGE. Proteins were separated in the second dimension by 12.5% acrylamide gel for 3–4 hours at 35mA per gel prior to staining with Silver Stain Plus (Bio-Rad).

IgIC immunoblots were performed with either whole cell lysates used for two-dimensional gels (1 µg total protein per lane) or from overnight bacterial broth cultures. For overnight cultures, 1.0 ml of was pelleted, washed, and resuspended in 100 µl of LDS loading buffer (Invitrogen), boiled for 5 minutes and sonicated. One µl of diluted (1:10) whole cell lysate were separated on a 12% acrylamide gel and transferred as stated above. Membranes were probed with a mouse anti-IgIC monoclonal antibody (clone 10D12, 1:20 dilution of hybridoma supernatant, Immuno-Precise Antibodies Ltd.) followed by an anti-mouse IgG antibody conjugated to HRP (1:15000, Sigma). Proteins were visualized using ECL chemiluminescent reagent (Amersham Biosciences) and exposure to film. Fold difference in protein amounts were calculated by comparing integrated density values (IDV) a BioRad Gel Doc system and Quantity One software (V4.4.0) (IDV = Σ(each pixel value-background pixel value)). Immunoblots were re-probed with a rabbit anti-hsp70 polyclonal antibody (1:750, GeneTex, Inc.) followed by an anti-rabbit IgG antibody conjugated to HRP (1:15000, Sigma), to examine protein load. Proteins were visualized using ECL and exposure to film as detailed above.

MALDI-TOF analysis

Protein spots of interest were picked manually (1.0 mm to 3.0 mm in diameter) and rinsed in 400 µl distilled water. Silver ions were removed by adding 50 µl fresh destaining solution (15

mM potassium ferricyanide and 50 mM thiosulfate in distilled water) (Invitrogen, Carlsbad, CA) to each spot and incubating for 20 min. Samples were then rinsed twice with 500 μ l distilled water and equilibrated with 100 mM ammonium bicarbonate (Fisher) for 20 min. Samples were rinsed 3 x in 400 μ l of 50% acetonitrile (Fisher) in 100 mM ammonium bicarbonate (23°C, 15 min) and dehydrated in 400 μ l of 100% acetonitrile (23°C, 10 min). Samples were digested in situ with 200–300 ng trypsin (Sigma) (14 hours, 37°C) and peptides were extracted twice with 50 μ l of 50% acetonitrile, 2.5% TFA (Fisher) in distilled water and dried using a CentriVap Speed Vacuum. Extracted, dried peptides were mixed with 5 μ l of α -cyano-4-hydroxycinnamic acid (CHCA) and 0.5 μ l was spotted onto the target for MALDI-TOF analysis. MALDI-TOF was performed using the 4700 Proteomics Analyzer (Applied BioSystems, Foster City, CA). The 4700 Proteomics Analyzer was primarily calibrated using the CalMix from ABI with the following instrument settings: *i*) minimum S/N of 10, *ii*) mass tolerance of $\pm$ 2 m/z, *iii*) minimum peaks to match of 6, *iv*) maximum outlier error of 5 ppm, and *v*) a laser intensity of 3900. Mass spectra were individually calibrated using internal trypsin peaks with Data Explorer software available from ABI. Proteins were identified using ProteinProspector (University of California, San Francisco; <http://prospector.ucsf.edu/>) set to a mass accuracy of $\pm$ 50 ppm to compare unknown mass fingerprints to those of known proteins in the NCBI database using a species-specific filter for *F. tularensis*.

Quantitative PCR

Bacteria were resuspended from chocolate II agar plates with RNAlater (Ambion) to preserve RNA content. Cells were then centrifuged and resuspended in 10ml TRI reagent (Molecular Research Center). RNA was isolated using a hot phenol extraction method according to the manufacturer's protocol. Samples were then treated with TURBOfree DNase (Ambion) and RNA quality was tested using an Agilent Bioanalyzer. cDNA synthesis was performed using Superscript III (Invitrogen) and 1 μ g of total RNA. Real time reactions were performed with a 1:5000 final dilution of template cDNA. *lux* primer sets (Invitrogen) or SYBR Green primer sets (Applied Biosystems) were designed for each of the genes of interest. The bacterial 16S rRNA gene was used as the internal reference and data were analyzed as expression in LVS relative to ACV.

Acknowledgments

We thank Dr. Karen Elkins (U.S. Food and Drug Administration) for providing *F. tularensis* LVS and *F. novicida* U112 stocks and Dr. Francis Nano (University of Victoria, British Columbia, Canada) for assistance in obtaining the anti-IgG monoclonal antibody. We thank the University of Pittsburgh Genomics and Proteomics Core facility for assisting with MALDI-TOF analysis. This research was supported by start up funds from the University of Pittsburgh School of Medicine. PEC received funding from training grant T32AI049820, "Molecular Microbial Persistence and Pathogenesis."

References

1. Ellis J, Oyston PC, Green M, Titball RW. Tularemia. Clin Microbiol Rev 2002;15:631–46. [PubMed: 12364373]
2. Dennis DT, Inglesby TV, Henderson DA, Bartlett JG, Ascher MS, Eitzen E, et al. Tularemia as a biological weapon: medical and public health management. Jama 2001;285:2763–73. [PubMed: 11386933]
3. Oyston PC, Quarry JE. Tularemia vaccine: past, present and future. Antonie Van Leeuwenhoek 2005;87:277–81. [PubMed: 15928980]
4. Elkins KL, Cowley SC, Bosio CM. Innate and adaptive immune responses to an intracellular bacterium, Francisella tularensis live vaccine strain. Microbes Infect 2003;5:135–42. [PubMed: 12650771]
5. Clemens DL, Lee BY, Horwitz MA. Virulent and avirulent strains of Francisella tularensis prevent acidification and maturation of their phagosomes and escape into the cytoplasm in human macrophages. Infect Immun 2004;72:3204–17. [PubMed: 15155622]

6. McCrumb FR. Aerosol infection of man with *Pasturella tularensis*. *Bacteriology Rev* 1961;25:267–267.
7. Saslaw S, Eigelsbach HT, Prior JA, Wilson HE, Carhart S. Tularemia vaccine study. II. Respiratory challenge. *Arch Intern Med* 1961;107:702–14. [PubMed: 13746667]
8. Bolger CE, Forestal CA, Italo JK, Benach JL, Furie MB. The live vaccine strain of *Francisella tularensis* replicates in human and murine macrophages but induces only the human cells to secrete proinflammatory cytokines. *J Leukoc Biol* 2005;77:893–7. [PubMed: 15758077]
9. Sandstrom G, Lofgren S, Tarnvik A. A capsule-deficient mutant of *Francisella tularensis* LVS exhibits enhanced sensitivity to killing by serum but diminished sensitivity to killing by polymorphonuclear leukocytes. *Infect Immun* 1988;56:1194–202. [PubMed: 3356465]
10. Gray CG, Cowley SC, Cheung KK, Nano FE. The identification of five genetic loci of *Francisella novicida* associated with intracellular growth. *FEMS Microbiol Lett* 2002;215:53–6. [PubMed: 12393200]
11. Nano FE, Zhang N, Cowley SC, Klose KE, Cheung KK, Roberts MJ, et al. A *Francisella tularensis* pathogenicity island required for intramacrophage growth. *J Bacteriol* 2004;186:6430–6. [PubMed: 15375123]
12. Golovliov I, Ericsson M, Sandstrom G, Tarnvik A, Sjostedt A. Identification of proteins of *Francisella tularensis* induced during growth in macrophages and cloning of the gene encoding a prominently induced 23-kilodalton protein. *Infect Immun* 1997;65:2183–9. [PubMed: 9169749]
13. Golovliov I, Sjostedt A, Mokrievich A, Pavlov V. A method for allelic replacement in *Francisella tularensis*. *FEMS Microbiol Lett* 2003;222:273–80. [PubMed: 12770718]
14. Lai XH, Golovliov I, Sjostedt A. Expression of IgIC is necessary for intracellular growth and induction of apoptosis in murine macrophages by *Francisella tularensis*. *Microb Pathog* 2004;37:225–30. [PubMed: 15519043]
15. Lauriano CM, Barker JR, Nano FE, Arulanandam BP, Klose KE. Allelic exchange in *Francisella tularensis* using PCR products. *FEMS Microbiol Lett* 2003;229:195–202. [PubMed: 14680699]
16. Lauriano CM, Barker JR, Yoon SS, Nano FE, Arulanandam BP, Hassett DJ, et al. MglA regulates transcription of virulence factors necessary for *Francisella tularensis* intramacrophage and intramacrophage survival. *Proc Natl Acad Sci U S A* 2004;101:4246–9. [PubMed: 15010524]
17. Lindgren H, Golovliov I, Baranov V, Ernst RK, Telepnev M, Sjostedt A. Factors affecting the escape of *Francisella tularensis* from the phagolysosome. *J Med Microbiol* 2004;53:953–8. [PubMed: 15358816]
18. Santic M, Molmeret M, Klose KE, Jones S, Kwaik YA. The *Francisella tularensis* pathogenicity island protein IgIC and its regulator MglA are essential for modulating phagosome biogenesis and subsequent bacterial escape into the cytoplasm. *Cell Microbiol* 2005;7:969–79. [PubMed: 15953029]
19. Cowley SC, Myltseva SV, Nano FE. Phase variation in *Francisella tularensis* affecting intracellular growth, lipopolysaccharide antigenicity and nitric oxide production. *Mol Microbiol* 1996;20:867–74. [PubMed: 8793882]
20. Lai XH, Golovliov I, Sjostedt A. *Francisella tularensis* induces cytopathogenicity and apoptosis in murine macrophages via a mechanism that requires intracellular bacterial multiplication. *Infect Immun* 2001;69:4691–4. [PubMed: 11402018]
21. Lai XH, Sjostedt A. Delineation of the molecular mechanisms of *Francisella tularensis*-induced apoptosis in murine macrophages. *Infect Immun* 2003;71:4642–6. [PubMed: 12874344]
22. Telepnev M, Golovliov I, Grundstrom T, Tarnvik A, Sjostedt A. *Francisella tularensis* inhibits Toll-like receptor-mediated activation of intracellular signalling and secretion of TNF- α and IL-1 from murine macrophages. *Cell Microbiol* 2003;5:41–51. [PubMed: 12542469]
23. Somerville GA, Chaussee MS, Morgan CI, Fitzgerald JR, Dorward DW, Reitzer LJ, et al. *Staphylococcus aureus* aconitase inactivation unexpectedly inhibits post-exponential-phase growth and enhances stationary-phase survival. *Infect Immun* 2002;70:6373–82. [PubMed: 12379717]
24. Feucht A, Evans L, Errington J. Identification of sporulation genes by genome-wide analysis of the sigmaE regulon of *Bacillus subtilis*. *Microbiology* 2003;149:3023–34. [PubMed: 14523133]
25. Oosthuizen MC, Steyn B, Theron J, Cosette P, Lindsay D, Von Holy A, et al. Proteomic analysis reveals differential protein expression by *Bacillus cereus* during biofilm formation. *Appl Environ Microbiol* 2002;68:2770–80. [PubMed: 12039732]

26. DeLisa MP, Wu CF, Wang L, Valdes JJ, Bentley WE. DNA microarray-based identification of genes controlled by autoinducer 2-stimulated quorum sensing in *Escherichia coli*. *J Bacteriol* 2001;183:5239–47. [PubMed: 11514505]
27. Chamberlain RE. Evaluation of Live Tularemia Vaccine Prepared in a Chemically Defined Medium. *Appl Microbiol* 1965;13:232–5. [PubMed: 14325885]
28. Bosio CM, Dow SW. *Francisella tularensis* induces aberrant activation of pulmonary dendritic cells. *J Immunol* 2005;175:6792–801. [PubMed: 16272336]
29. Hubalek M, Hernychova L, Havlasova J, Kasalova I, Neubauerova V, Stulik J, et al. Towards proteome database of *Francisella tularensis*. *J Chromatogr B Analyt Technol Biomed Life Sci* 2003;787:149–77.
30. Hernychova L, Stulik J, Halada P, Macela A, Kroca M, Johansson T, et al. Construction of a *Francisella tularensis* two-dimensional electrophoresis protein database. *Proteomics* 2001;1:508–15. [PubMed: 11681204]
31. Havlasova J, Hernychova L, Halada P, Pellantova V, Krejsek J, Stulik J, et al. Mapping of immunoreactive antigens of *Francisella tularensis* live vaccine strain. *Proteomics* 2002;2:857–67. [PubMed: 12124931]
32. Lenco J, Pavkova I, Hubalek M, Stulik J. Insights into the oxidative stress response in *Francisella tularensis* LVS and its mutant DeltaiglC1+2 by proteomics analysis. *FEMS Microbiol Lett* 2005;246:47–54. [PubMed: 15869961]
33. Hubalek M, Hernychova L, Brychta M, Lenco J, Zechovska J, Stulik J. Comparative proteome analysis of cellular proteins extracted from highly virulent *Francisella tularensis* ssp. *tularensis* and less virulent *F. tularensis* ssp. *holarctica* and *F. tularensis* ssp. *mediaasiatica*. *Proteomics* 2004;4:3048–60. [PubMed: 15378745]
34. Somerville G, Mikoryak CA, Reitzer L. Physiological characterization of *Pseudomonas aeruginosa* during exotoxin A synthesis: glutamate, iron limitation, and aconitase activity. *J Bacteriol* 1999;181:1072–8. [PubMed: 9973331]
35. Twine SM, Mykytczuk NC, Petit MD, Shen H, Sjostedt A, Wayne Conlan J, et al. In vivo proteomic analysis of the intracellular bacterial pathogen, *Francisella tularensis*, isolated from mouse spleen. *Biochem Biophys Res Commun* 2006;345:1621–33. [PubMed: 16730660]
36. Maki Y, Yoshida H, Wada A. Two proteins, YfiA and YhbH, associated with resting ribosomes in stationary phase *Escherichia coli*. *Genes Cells* 2000;5:965–74. [PubMed: 11168583]
37. Juhas M, Eberl L, Tummli B. Quorum sensing: the power of cooperation in the world of *Pseudomonas*. *Environ Microbiol* 2005;7:459–71. [PubMed: 15816912]
38. Cvitkovitch DG, Li YH, Ellen RP. Quorum sensing and biofilm formation in Streptococcal infections. *J Clin Invest* 2003;112:1626–32. [PubMed: 14660736]
39. Stritzker J, Schoen C, Goebel W. Enhanced synthesis of internalin A in *aro* mutants of *Listeria monocytogenes* indicates posttranscriptional control of the *inlAB* mRNA. *J Bacteriol* 2005;187:2836–45. [PubMed: 15805530]
40. Pessi G, Williams F, Hindle Z, Heurlier K, Holden MT, Camara M, et al. The global posttranscriptional regulator RsmA modulates production of virulence determinants and N-acylhomoserine lactones in *Pseudomonas aeruginosa*. *J Bacteriol* 2001;183:6676–83. [PubMed: 11673439]
41. Tomich M, Mohr CD. Transcriptional and posttranscriptional control of cable pilus gene expression in *Burkholderia cenocepacia*. *J Bacteriol* 2004;186:1009–20. [PubMed: 14761995]
42. Jude F, Kohler T, Branny P, Perron K, Mayer MP, Comte R, et al. Posttranscriptional control of quorum-sensing-dependent virulence genes by DksA in *Pseudomonas aeruginosa*. *J Bacteriol* 2003;185:3558–66. [PubMed: 12775693]
43. Cherwonogrodzky JW, Knodel MH, Spence MR. Increased encapsulation and virulence of *Francisella tularensis* live vaccine strain (LVS) by subculturing on synthetic medium. *Vaccine* 1994;12:773–5. [PubMed: 7975855]
44. Loegering DJ, Drake JR, Banas JA, McNealy TL, McArthur DG, Webster LM, et al. *Francisella tularensis* LVS grown in macrophages has reduced ability to stimulate the secretion of inflammatory cytokines by macrophages in vitro. *Microb Pathog.* 2006
45. Deng K, Blick RJ, Liu W, Hansen EJ. Identification of *Francisella tularensis* genes affected by iron limitation. *Infect Immun* 2006;74:4224–36. [PubMed: 16790797]

46. Carroll JA, Garon CF, Schwan TG. Effects of environmental pH on membrane proteins in *Borrelia burgdorferi*. *Infect Immun* 1999;67:3181–7. [PubMed: 10377088]
47. Markwell MA, Haas SM, Bieber LL, Tolbert NE. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal Biochem* 1978;87:206–10. [PubMed: 98070]
48. Nowalk AJ, Nolder C, Clifton DR, Carroll JA. Comparative proteome analysis of subcellular fractions from *Borrelia burgdorferi* by NEPHGE and IPG. *Proteomics* 2006;6:2121–34. [PubMed: 16485259]

Figure 1. Proinflammatory cytokine production by human monocyte derived macrophages following exposure to *F. tularensis* LVS and ACV

Macrophages were cultured with either LVS or ACV at an MOI of 10 for 24 hours. Levels of TNFα (A.), IL-1β (B.), IL-6 (C.), and IL-12 p40 (D.) were measured in supernatants by ELISA. Data are mean ± SD of triplicate wells within one experiment. Data are representative of 5 individual experiments with different donors. * indicates cytokine levels below detectable limits of the ELISA. Bacteria were grown in MH broth to an OD₆₀₀ of 0.2 for LVS and > 1.0 for ACV.

Figure 2. Macrophage response to live bacteria and bacterial lysates

(A.) TNF α production by human monocyte derived macrophage exposed to a range of MOIs of LVS (◆, black lines) or ACV (■, gray lines) for 24 hours. Data are mean $\pm$ SD of triplicate wells within one experiment. (B.) Decreased cytokine response to LVS requires live, intact bacteria. Proteins lysates from each variant stimulate macrophage TNF α production. Data are mean $\pm$ SD of triplicate wells within one experiment. Cytokine levels were measured by ELISA and similar results were seen in macrophages from 3 separate donors.

Figure 3. Inhibition of macrophage TLR signaling by LVS

Macrophages were incubated with either media, LVS, or ACV for 4 hours (MOI = 500), washed, and treated with LPS for 20 hours. Following incubation, supernatants were harvested and levels of TNFα were measured using ELISA. Data are mean $\pm$ SD of triplicate wells within one experiment and are representative of experiments performed on two separate donors. * denotes $p < 0.002$.

Figure 4. Immunoreactivity of LPS from LVS and ACV

Proteinase K treated whole cell lysates from LVS and ACV were separated on a 4–12% Bis-Tris gradient gel by SDS-PAGE and transferred to PVDF membrane. After blocking, membranes were probed with anti- *F. tularensis* LVS or anti- *F. novicida* LPS monoclonal antibodies followed by anti-mouse IgG conjugated to Alexa Fluor 488 for detection. LPS from both LVS isolates reacted with anti-*F. tularensis* LPS monoclonal antibody (right) and anti-*F. novicida* LPS monoclonal antibody (left).

Figure 5. Differences in protein expression between LVS and ACV

Whole cell lysates or subcellular fractions of LVS and ACV were separated on a one-dimensional SDS-PAGE gel (12.5% acrylamide). The gel was stained with silver reagents. Cellular fractions are labeled: CL = cell lysate, MP = membrane proteins, and SP = soluble proteins. Apparent differences of protein content in the soluble fraction are indicated with arrows.

Figure 6. Identification of differences in the soluble protein fractions of LVS and ACV by two-dimensional gel electrophoresis

Soluble protein fractions of LVS and ACV were separated in first dimension by isoelectric focusing and by size in second dimension on a 12.5% acrylamide gel. Silver-stained gels contained multiple differentially-expressed proteins between LVS and ACV. Specific proteins are numbered and are identified in Table 1. Only those spots reproducibly different in two different protein isolations and on five repeated gels are labeled. Positions of molecular mass standards (kDa) and pI range are indicated on the left and bottom, respectively.

Figure 7. Quantification of IgIC levels by immunoblot

Whole cell lysates from LVS and ACV were separated by SDS-PAGE (12% acrylamide) and transferred to PVDF membrane. After blocking, the membrane was probed for IgIC expression (top), using monoclonal mouse anti-IgIC and anti-mouse IgG conjugated to HRP, and then re-probed with polyclonal anti-Hsp70 to assess protein loads (bottom). Levels of IgIC and Hsp70 expression were quantified using densitometry. IgIC expression was 15-fold higher in LVS samples, while Hsp70 controls exhibited less than 1.5-fold differences.

Figure 8. ACV reversion to the LVS phenotype

(A.) Schematic representation of strains and growth conditions used in reversion experiments. LVS and ACV were grown in CDM or MH to a high density ($A_{600} \geq 1.0$). LVS grown in CDM or MH are designated “LVS CDM” and “LVS MH.” Similarly, ACV grown in CDM or MH are designated “ACV CDM” and “ACV MH.” Bacteria were harvested from these overnight cultures and were used for the experiments described in B and C. (B.) ACV induced low levels of TNF α following growth in CDM. Macrophages were incubated with media only (“media”) or in the presence of the four bacterial cultures at an MOI of 10 for 24 hours. TNF α levels were measured by ELISA. Data are mean $\pm$ SD of triplicate wells within one experiment. Data are representative of 3 individual experiments with macrophages derived from different donors. (C.) IgIC protein expression increased following growth of ACV in CDM. Aliquots of the cultures used in part B were lysed using LDS loading buffer (Invitrogen). After SDS-PAGE of whole cell lysates and transfer, the PVDF membrane was probed for IgIC expression (top) and then re-probed for Hsp70 levels to assess protein loads (bottom). IgIC levels were 30 and 60 fold greater in LVS and ACV, respectively, following growth in CDM. Hsp70 loading controls exhibited less than 1.5 fold differences.

Table 1

Macrophage viability following incubation with LVS isolates.

Sample	% max LDH release ^{a,b}		Live (SYTO9)/Dead (PI) ^{a,b}	
	Mean	std. dev	Mean	std. dev
Media	12.25	± 1.73	18.65	± 0.70
LVS	14.02	± 2.19	17.7	± 0.21
ACV	13.26	± 3.54	17.73	± 0.23

^aData are representative of three experiments on separate donors.^bOther experiments showed modest elevations of LDH after bacterial culture, though no differences were seen between the treatment groups.

Table 2
MALDI-TOF identification of proteins present in LVS but reduced in ACV

Spot	Identification (locus designation)	% Cov. ^a	MP ^b	Theoretical ^c		Observed	
				pI	mass(Da)	pI	mass(Da)
1	Aconitate hydratase, Aconitase, <i>acnA</i> homolog (FTT0087)	37	42	5.4	102,615	5.4	102,450
2	Fumerate hydratase, <i>fumA</i> homolog (FTT1600c)	45	26	5.2	54,974	5.2	58,340
3	Intracellular growth locus subunit C, <i>iglC</i> (FTT1712c or FTT1357c)	37	9	6.3	22,433	5.6	22,000
4	Intracellular growth locus subunit C, <i>iglC</i> (FTT1712c or FTT1357c)	40	11	6.3	22,433	5.6	21,590
5	Heat shock protein, <i>hsp20</i> homolog (FTT1794)	33	5	5.7	16,712	5.7	14,410
6	Sigma-54 modulation protein, <i>yhbH</i> homolog (FTT1281c)	46	8	6.3	11,182	6.3	12,660

^aPercent coverage of the identified protein
^bNumber of matching peptides
^cObtained from the TIGR website and does not include post translational modifications.