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In vivo proteomic analysis of the intracellular bacterial pathogen, *Francisella tularensis*, isolated from mouse spleen

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Abstract

Understanding the pathogenesis of infectious diseases requires comprehensive knowledge of the proteins expressed by the pathogen during in vivo growth in the host. Proteomics provides the tools for such analyses but the protocols required to purify sufficient quantities of the pathogen from the host organism are currently lacking. Here, we present a rapid immunomagnetic protocol for the separation of *Francisella tularensis*, a highly virulent bacterium and potential biowarfare agent, from the spleens of infected mice. In less than one hour, bacteria can be isolated in quantities sufficient to carry out meaningful proteomic comparisons with in vitro grown bacteria. Furthermore, the isolates are virtually free from contaminating host proteins. Two-dimensional gel analysis revealed a host induced proteome in which 78 proteins were differentially expressed in comparison to in vitro grown controls. The results obtained clearly demonstrate the complexity of the adaptive response of *F. tularensis* to the host environment, and the difficulty of mimicking such behavior in vitro.

Keywords: Francisella tularensis; Proteomics; Mice; Immunomagnetic separation; Two-dimensional gel electrophoresis; In vivo; Immunoproteomics

Francisella tularensis is one of the most virulent microbes known and the causative agent of tularemia. Experiments with human volunteers showed that as few as 10 cfu of type A *F. tularensis* can initiate infection and represents a lethal dose for various experimental animals; this is assumed to be the case also for humans [1,2]. *F. tularensis* can be disseminated by aerosol to initiate both transdermal and respiratory infection [3]. The low infective dose and ability to be disseminated by aerosol led to *F. tularensis* being developed as a biowarfare agent by various governments [4]. Recent world events have led to renewed concern about its possible abuse as a weapon of mass destruction by terrorists [4].

Little is known about the molecular basis for the extreme virulence of type A *F. tularensis*. However, its capacity to cause disease appears to be a reflection of its ability to multiply intracellularly within and damage various host organs rather than its ability to produce any specific toxins. This requires *F. tularensis* to subvert or otherwise avoid a variety of host defenses that possess the potential to kill it. In particular, *F. tularensis* multiplies extensively in macrophages in vitro and in vivo [5,6]. Macrophages normally kill the microbial pathogens they ingest. Therefore, *F. tularensis* must have evolved particular survival mechanisms to flourish in this usually hostile host environment. In this regard, earlier studies using an attenuated strain, *F tularensis* LVS, of the less virulent *holarctica* subspecies (type B subspecies) showed that it upregulated several proteins when grown in macrophages [5]. These proteins were also induced by exposing LVS to the macrophage microbicide, hydrogen peroxide (H_2O_2)

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[3]. In vivo, sequential inflammatory changes occur at sites of *Francisella* infection, including the accumulation of phagocytic neutrophils and monocytes, and lymphocytes that can potentially activate macrophages to kill the pathogen [7]. It is reasonable to suppose that such events might, in turn, influence the expression of pathogen-protective mechanisms including proteins.

Currently, it is impossible to mimic in vitro the myriad of often unknown, inflammatory events that occur following in vivo parasitization of macrophages by *F. tularensis*. Therefore, in the present study we developed an immunomagnetic isolation method for the rapid purification of virulent type A *F. tularensis* from the spleens of infected mice. The technique is effective and bacteria can be isolated in quantities sufficient for comparative proteomic analyses by 2D-gel electrophoresis (2DE). Furthermore, the bacterial isolates have very low levels of contaminating host proteins. Proteins extracted from the in vivo isolates were compared with extracts from in vitro cultures with and without exposure to H_2O_2. For the first time, therefore, it was possible to examine the proteome expressed by this highly virulent pathogen in the host environment. The results showed that a far different proteome was expressed by the pathogen in vivo than in vitro even after exposure of the latter organisms to H_2O_2 stress.

Materials and methods

In vitro growth of F. tularensis. Type A *F. tularensis* strain FSC033 was originally isolated from a squirrel in Georgia, USA [8], and is archived in the *Francisella* strain collection (FSC) of the Swedish Defence Research Agency, Umeå, Sweden. We have used it extensively for *F. tularensis* infection and immunity studies over the past several years (e.g. [9–13]). Frozen stocks were prepared as previously described [14]. For in vitro studies, 0.10 mL of thawed stock FSC033 was inoculated into 100 mL of modified Mueller–Hinton broth and grown overnight at 37 °C with shaking. Then, H_2O_2 was added to a final concentration of 5 mM and flasks incubated for a further 45 min. In control cultures sterile deionized water substituted for H_2O_2. Aliquots were removed pre- and post-treatment for viable cell counts. Bacteria were harvested by centrifugation and washed twice with ice-cold sterile Milli-Q water. The experiment was performed on three separate occasions.

In vivo growth of F. tularensis. Specific-pathogen-free female BALB/c mice were purchased from Charles Rivers Laboratories (St. Constant, Que.). Mice were maintained and used in accordance with the recommendations of the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals. Intradermal (i.d.) inocula (50 μL/mouse) of 10 cfu of FSC033 were injected into the shaved mid-belly. In our hands this initiates a uniformly lethal infection that kills mice between day 5 and 6 [6,7]. Therefore, for the current study mice were killed on day 4 of infection, by which time infected organs (lung, liver, and spleen) harbor 10^8–10^9 cfu of the pathogen. The spleen atrophies during the terminal stages of this infection increasing the ratio of bacterial:host tissue mass and is also easy to disrupt by homogenization. Thus, this organ represents the most amenable site from which to attempt to purify in vivo grown bacteria in sufficient quantities for proteomic analysis.

Purification of bacteria from spleen. Bacteria were isolated from homogenized spleen tissues by immunomagnetic separation (IMS). Dynabeads™ M280 (Dynal, Oslo, Norway) supplied with covalently attached sheep anti-rabbit IgG were labeled with rabbit-anti-*Francisella* antisera (BD Biosciences, Maryland, USA) as per the manufacturer's instructions. Briefly, 1 mL aliquots of magnetic beads were incubated with 300 μL of rabbit antisera overnight at room temperature on a 360° rotator. Beads were magnetically separated from the labeling mixture and washed in phosphate-buffered saline (PBS) containing 0.1% (v/v) bovine serum albumin (BSA) prior to use in magnetic separations.

For each experiment, spleens from 4 to 6 mice were removed aseptically and homogenized for 15 s using an aerosol-proof homogenizer. Five independent experiments were conducted over a 12 month period. In each case, individual spleen homogenates were pooled prior to magnetic separations. Viable cell counts were determined before and after magnetic separation of bacteria from host tissues by plating 10-fold serial dilutions. Following homogenization, the spleen suspensions were passed through a 70 μm filter and diluted 1:1 with ice-cold PBS containing BSA. Next, 1000 μL of Dynabeads™ M280 labeled with normal rabbit serum (Vector Labs) was added as a blocking step, and the suspension was incubated for 10 min. with constant mixing. Beads were removed using a magnet, and the supernatant was transferred to another tube containing 500–1000 μL of Dynabeads™ M280 labeled with rabbit anti-*Francisella* antisera. The mixture was incubated for 15–20 min. with constant rotation. The beads-bacteria complex was then isolated by magnet and resuspended to homogeneity in 50 mM Hepes, pH 7.0. The beads-bacteria complex was washed twice more with Hepes and once with high-purity water (Millipore Milli-Q Plus, Millipore Corp., Billerica, MA). Bacteria were eluted from the beads by the addition of 1000 μL of 0.2 M sodium citrate, pH 2.3, and pelleted by centrifugation. Pelleted bacteria were immediately resuspended in lysis buffer. The entire process of bacterial purification was completed in less than 60 min. Controls included sterile spleen homogenates and homogenates spiked with in vitro grown *F. tularensis* subjected to the same separation strategy as above.

Two-dimensional gel electrophoresis. Preparation of bacterial soluble protein extracts and two-dimensional separations were carried out as previously described [14]. Gel image analysis was carried out using PDQuest software. Matchsets were created for 2DE in the pH ranges 4–7 and 6–11, with each matchset consisting of 8 Sypro Ruby (sensitive to 1 ng protein) stained 2DE, four gels from each for control and H_2O_2-stressed or spleen-purified samples, with at least one gel included from each of three biological repeats. Spots were detected and matched using PDQuest software, checked manually, and corrected when required. Gel-to-gel variation in spot number was expressed as % coefficient of variation (% CV = 100 × (standard deviation/mean number of spots detected in gel set)). The normalized intensity for each spot was calculated as the ratio of the spot intensity versus the sum of the intensities of all spots present in the gel. Variations in spot intensity were calculated as the ratio of normalized intensities for each spot in the gels. In some cases the spot intensity was below 200 ppm and was considered below the limits of detection. Normalized values of spot intensity were used to calculate the ratio of spot intensities. Spots exhibiting a 2-fold or greater intensity difference between samples undergoing comparison and with a p value <0.05 (Student's t-test) were considered to be differentially expressed. All spots considered to be differentially expressed were then checked manually against matchset gels to ensure correct matching and valid spot assignments. Where spots could not be matched, a list of unmatched spots was generated and verified manually. Only those spots with an intensity >200 ppm found in all gels of one gel set and absent in all gels of the second gel set were accepted as unmatched.

For immunoblotting, proteins separated by 2DE were transferred to PDVF membranes (Immobilon-P, 0.45 mm, Millipore, Billerica, MA, USA) using established methods [15]. Briefly, proteins were electroblotted at 15 V for 1 h using Trans Blot Cell (Bio-Rad, Hercules, CA). PDVF membranes were incubated overnight with PBST (9 mM sodium phosphate, 0.15 M NaCl, and 0.05 % v/v Tween 20) with 5% w/v skimmed milk powder at 4 °C with constant rotation. Following three 10 min washes with PBST, the PVDF membrane was incubated with mouse anti-*Francisella* serum diluted 1:1000. Incubation was for 1 h at room temperature with constant rotation. After washing with PBST, blots were then incubated with the peroxidase-conjugated goat anti-mouse immunoglobulin for 1 h at room temperature. Reactive spots were visualized using the Western Lightning Chemilumiscence kit (Perkin-Elmer Life Sciences, Boston, USA) and image captured using a Fluor-S Imager (Bio-Rad,