Possible Links Between Stress Defense and the Tricarboxylic Acid (TCA) Cycle in Francisella Pathogenesis

Jennifer Dieppedale‡‡§§, Gael Gesbert‡‡§§, Elodie Ramond‡‡§§*, Cerina Chhuon‡¶¶*, Iharilalo Dubait‡¶¶, Marion Dupuis‡¶¶, Ida Chiara Guerrera‡¶¶, and Alain Charbit‡‡§§

Francisella tularensis is a highly infectious bacterium causing the zoonotic disease tularemia. In vivo, this facultative intracellular bacterium survives and replicates mainly in the cytoplasm of infected cells. We have recently identified a genetic locus, designated moxR that is important for stress resistance and intramacrophage survival of F. tularensis. In the present work, we used tandem affinity purification coupled to mass spectrometry to identify in vivo interacting partners of three proteins encoded by this locus: the MoxR-like ATPase (FTL_0200), and two proteins containing motifs predicted to be involved in protein–protein interactions, bearing von Willebrand A (FTL_0201) and tetratricopeptide (FTL_0205) motifs. The three proteins were designated here for simplification, MoxR, VWA1, and TPR1, respectively. MoxR interacted with 31 proteins, including various enzymes. VWA1 interacted with fewer proteins, but these included the E2 component of 2-oxoglutarate dehydrogenase and TPR1. The protein TPR1 interacted with one hundred proteins, including the E1 and E2 subunits of both oxoglutarate and pyruvate dehydrogenase enzyme complexes, and their common E3 subunit. Remarkably, chromosomal deletion of either moxR or tpr1 impaired pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase activities, supporting the hypothesis of a functional role for the interaction of MoxR and TPR1 with these complexes. Altogether, this work highlights possible links between stress resistance and metabolism in F. tularensis virulence.

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Francisella tularensis is responsible for the disease tularemia in a large number of animal species. This highly infectious bacterial pathogen can be transmitted to humans in numerous ways (1, 2, 3), including direct contact with sick animals, inhalation, ingestion of contaminated water or food, or by bites from ticks, mosquitoes, or flies. Four different subspecies (subsp.) of F. tularensis that differ in virulence and geographic distribution exist, designated subsp. tularensis (type A), subsp. holarctica (type B), subsp. Novicida, and subsp. mediasiatica, respectively. F. tularensis subsp. tularensis is the most virulent subspecies causing a severe disease in humans, whereas F. tularensis subsp. holarctica causes a similar disease but of less severity (4). Because of its high infectivity and lethality, F. tularensis is considered a potential bioterrorism agent (5).

F. tularensis is able to survive and to replicate in the cytoplasm of a variety of infected cells, including macrophages. To resist this stressful environment, the bacterium must have developed stress resistance mechanisms, most of which are not yet well characterized. We recently reported the identification of a novel genetic locus that is important for stress resistance and intracellular survival of F. tularensis (6). This locus was designated moxR because the first gene FTL_0200, encodes a protein belonging to the AAA+ ATPase of the MoxR family ((7) and references therein). The data obtained in that first study had led us to suggest that the F. tularensis MoxR-like protein might constitute, in combination with other proteins of the locus, a chaperone complex contributing to F. tularensis pathogenesis.

To further validate this hypothesis and expand our initial observations, we here decided to perform tandem affinity purification (TAP), using a dual affinity tag approach coupled to mass spectroscopy analyses (8), to identify proteins interacting with fewer proteins, but these included the E2 component of 2-oxoglutarate dehydrogenase and TPR1. The protein TPR1 interacted with one hundred proteins, including the E1 and E2 subunits of both oxoglutarate and pyruvate dehydrogenase enzyme complexes, and their common E3 subunit. Remarkably, chromosomal deletion of either moxR or tpr1 impaired pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase activities, supporting the hypothesis of a functional role for the interaction of MoxR and TPR1 with these complexes. Altogether, this work highlights possible links between stress resistance and metabolism in F. tularensis virulence.

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acting in vivo with three proteins encoded by the proximal portion of the moxR locus. For this, we chose as baits: the MoxR-like protein (FTL_0200) and two proteins bearing distinct motifs possibly involved in protein–protein interactions, FTL_0201 (Von Willebrand Factor Type A domain, or VWA) and FTL_0205 (tetratricopeptide repeat or TPR). The three proteins were designated here for simplification, MoxR, VWA1, and TPR1; and the corresponding genes moxR, vwa1, and tpr1, respectively.

VWA domains are present in all three kingdoms of life. They consist of a β-sheet sandwiched by multiple α-helices. Frequently, VWA-domain-containing proteins function in multiprotein complexes (9). TPR typically contain 34 amino acids. Many three-dimensional structures of TPR domains have been solved, revealing amphipathic helical structures (10). TPR-containing proteins are also found in all kingdoms of life. They can be involved in a variety of functions, and generally mediate protein–protein interactions. In the past few years, several TPR-related proteins have been shown to be involved in virulence mechanisms in pathogenic bacteria (11) and references therein.

Our proteomic approach allowed us to identify a series of protein interactants for each of the three moxR-encoded proteins. Remarkably, the protein TPR1 interacted with all the subunits of the pyruvate dehydrogenase (PDH) and 2-oxoglutarate dehydrogenase (OGDH) complexes. Furthermore, inactivation of tpr1 also severely impaired the activities of these two enzymes. Inactivation of tpr1 affected bacterial resistance to several stresses (and in particular oxidative stress), intramacrophagic bacterial multiplication and bacterial virulence in the mouse model. Functional implications and possible relationship between bacterial metabolism, stress defense, and bacterial virulence are discussed.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids—Francisella tularensis** LVS was grown on premed chocolate agar (BioMerieux SA Marcy l’Etoile, France), chocolate plates prepared from GC medium base, IsoVitalex vitamins, and hemoglobin (BD Biosciences, San Jose, CA, USA), or in Schaedler + vitamin K3 broth (Schaedler-K3, BioMerieux) at 37 °C. All bacterial strains, plasmids, and primers used in this study are listed in supplemental Table S1. The construction of a chromosomal LVSΔtpr1 deletion mutant—The F. tularensis LVSΔtpr1 deletion mutant was constructed using the same procedure as described previously (6). Briefly, we used the <suicide> plasmid pPV an E. coli-F. tularensis shuttle vector (12), which possesses a sacB counter-selective gene, chloramphenicol and amphicillin selective genes, an E. coli replication origin (ori pUC19), and a transfer origin for conjugation (oriT RP4). The upstream and downstream regions of tpr1 gene were amplified by overlapping PCR, and the resulting fragment was subcloned in the XbaI and SalI restriction sites of the plasmid pPV, yielding recombinant pPV-Δtpr1. This plasmid was introduced in LVS by conjugation (6) and the chromosomal Δtpr1 deletion mutant was obtained by the classical two-step allelic replacement procedure (12).

**Functional Complementation—Plasmids** pFNLT6p6gro-tpr1 and pFNLT6p6gro-tpr1-FTL_0206 (here designated p6gro-tpr1 and p6gro-tpr1-FTL_0206) were used for complementation of LVSΔtpr1. Plasmid p6gro-tpr1 was constructed by amplifying a 761 base pair (bp) fragment comprising the sequence 16 bp upstream of the tpr1 start codon to 11 bp downstream of the stop codon. The primers used were Compl-FTL_0205-FW and Compl-FTL_0205-RV (supplemental Table S1). The plasmid p6gro-tpr1-FTL_0206, was constructed by amplifying a 2508 bp fragment (corresponding to the sequence 100 bp upstream of the tpr1 start codon and to 39 bp downstream of the stop codon of the FTL_0206 gene) using primers Compl-FTL_205/206-FW EcoRI and Compl-FTL_205/206-RV BamHI (supplemental Table S1), followed by digestion with EcoRI and BamHI, and cloning into plasmid pFNLT66p6gro (p6gro) (13). The amplified product was digested with BamHI and EcoRI and cloned into the corresponding sites of plasmid p6gro.

The plasmids p6gro (plasmid without insert), p6gro-tpr1 and p6gro-tpr1-FTL_0206 (the complementing plasmids) were introduced into LVS or LVSΔtpr1 mutant strain by electroporation, as described previously (6).

**Construction of Strains Expressing TAP-tagged Proteins—The** moxR, vwa1 and tpr1 genes were amplified from genomic DNA of LVS, using the appropriate pairs of primers (supplemental Table S1). The amplified fragments were cloned into the expression vector p6gro, resulting in the following plasmids: p6gro-moxR, p6gro-vwa1, p6gro-tpr1. The tandem affinity purification tag (TAP) tag sequence was amplified from plasmid pEB304 (8, 14), using primers 7 or 8, and 9 (supplemental Table S1). This TAP tag sequence (composed of the sequence of protein A, a TEV protease cleavage site, and the calmodulin binding domain) was fused in frame, in the Nofl or Xhol restriction sites, after the last codon of each coding sequence) to the C-terminal end of each gene (in p6gro recombinant plasmids). The resulting plasmids were finally introduced in the LVS strain by electroporation, yielding the recombinant strains LVS/p6gro-moxR-TAP, LVS/p6gro-vwa1-TAP, and LVS/p6gro-tpr1-TAP, respectively. The tagged proteins were designated MoxR-TAP, VWA1-TAP, and TPR1-TAP, respectively. The p6gro-moxR-TAP plasmid was also introduced in the mutant strain LVSΔtpr1 to verify the activity of protein TPR1-TAP by functional complementation.

**Purification of TAP-tagged Proteins from F. tularensis LVS—F. tularensis** cells of logarithmic phase (2 liters of culture at an OD600 of 0.5–0.7) were harvested (3000 × g for 20 min) and resuspended in 20 ml of a solubilization buffer consisting of 100 mM HEPES/KOH, pH 7.4, 100 mM KCl, 8% glycerol and complete protease inhibitor mixture, Roche (one tablet for 50 ml of buffer). Then, cells were harvested (3000 × g for 30 min) and resuspended in 10 ml of solubilization buffer. The cross-linker dithiobis(succinimidyl propionate) (DSP) (G-Biosciences) was added at a concentration of 20 mg/ml for 5 min on ice, and the cross-link reaction was stopped by addition of 1 mM Tris/HCl pH 7.4 (15). The bacterial suspension was sonicated 20 times for 30 s at 4.0 output, 70% pulses (Branson Sonifier 250). After centrifugation of cell debris (16,000 × g for 30 min), 0.1% Nonidet P-40 was added to the protein lysate. Then, the protein extracts were incubated for 2 h at 4 °C, with 0.5 ml of Sepharose-IGG beads (GE Healthcare), previously washed with binding buffer (100 mM HEPES/ KOH, pH 7.4, 100 mM KCl, 8% glycerol, 0.1% Nonidet P-40). The column was washed three times with binding buffer. The bound proteins were recovered by addition of 1 ml (four times with 250 μl) of 100 mM glycine-HCl at pH 3, supplemented with 100 mM KCl. The eluate was added to three volumes of Calmodulin binding buffer (binding buffer supplemented with 4 mM CaCl2) and the pH was readjusted to 7 by addition of KOH before binding to the calmodulin beads. Then, this protein eluate was incubated at 4 °C for 1 h with 0.5 ml of Sepharose-calmodulin beads (GE Healthcare), previously washed with calmodulin binding buffer. After, the column was washed three times with this same buffer and the protein complex was eluted with 1 ml (five times with 200 μl) of 20 mM Tris/HCl pH 8,
containing 50 mM NaCl, 5 mM EGTA. Purified proteins were washed with PBS 1X and concentrated, using microcon 10,000 MWCO (Amicon, Millipore). Proteins were quantified with BCA assay kit (Pierce, Thermo Scientific).

Western Blot Analysis—The TAP-tagged proteins were detected by Western blot using ECL™ Western blot Detection Reagents (GE Healthcare) and anti-TAPtag antibodies (Genscript), according to the manufacturer’s recommendations. The primary rabbit anti-TAPtag antibody was used at a final dilution of 1:1000 in PBS Tween 0.05%, 5% milk (PBS-TM), and incubated for 1 h. The secondary anti-rabbit antibody was used at a final dilution of 1:2000 in PBS-TM and incubated for 1 h.

Bacterial Two-Hybrid (BACTH) Complementation Assays—The LVS genes were amplified from genomic DNA, using the appropriate pairs of primers (supplemental Table S1). These genes were fused in-frame to the DNA sequences encoding the T18 or T25 domains of Bordetella pertussis adenylate cyclase. The gene moxR was cloned into pKT25 vector (C-terminal fusion to T25 domain), and the gene tpr1 was cloned into pKT25 and pKTN25 vectors (yielding C-terminal and N-terminal fusions to T25, respectively) (16). The genes TFL_1783, TFL_0309, TFL_0310, TFL_0311, and TFL_0295, were cloned into pUT18C vector (C-terminal fusion to T18 domain). The different pairs of recombinant plasmids (pKT25-x/pUT18C-y or pKTN25-x/pUT18C-y) were used to transformat E. coli strain BTH101.

The transformants were selected onto LB plates containing 40 µg/ml X-Gal, 0.5 mM IPTG, 50 µg/ml Kanamycin and 100 µg/ml Ampicillin, and incubated at 30 °C for 24 to 36 h. As negative control, BTH101 cells were co-transformed with empty pUT18C vector and pKT25 vector containing either moxR gene or tpr1 gene (or pKTN25 vector containing tpr1 gene). Each BACTH assay was performed twice.

Enzymatic Assays—LVS, LVSΔmoxR, and LVSΔtpr1 strains were grown until exponential phase (OD600 of 0.5–0.7). The cells (15 ml) were harvested by centrifugation at 3000 × g for 20 min and resuspended in 2 ml of 5 mM Tris-HCl pH 8. The bacterial suspensions were sonicated four times for 30 s at 4.0 output, 70% pulsed (Branson Sonifier 250). Oxoglutarate and pyruvate dehydrogenase activities were measured by reducing the measurement of NAD− at 340 nm upon the addition of 0.5 mM NAD+, 200 µM TPP, 40 µM CoASH, 3 mM MgCl2, 9 mM l-cysteine and either 16 mM oxoglutarate (κ-ketoglutarate) or 16 mM pyruvate to 20 µM l−1. F. tularensis cell lysate, as described in (17, 18). Protein concentration was estimated with BCA assay kit (Pierce, Thermo Scientific). Assays were performed in a final volume of 1 ml.

Assays for the carboxyltransferase subunit of acetyl-CoA carboxylase were performed at 412 nm in the reverse direction in which malonyl-CoA reacts with biocytin to form acetyl-CoA and carboxybiotin. The production of acetyl-CoA was coupled to citrate synthase, which produced citrate and coenzyme A. The amount of coenzyme A formed was detected using 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), as described in (19).

The assay buffer consisted of 100 mM potassium phosphate (pH 7.6) with 0.1% Tween 20. Final concentrations of reagents in the assay were as follows: 200 mM malonyl-CoA, 12 mM biocytin, 10 mM oxaloacetate, 200 mM DTNB, 104 mM citrate synthase, and 50 µg of total protein. The assay was realized in a final volume of 100 µl in 96-well plates, with a classic plate reader (Multiskan RC, ThermoLabsystems).

Catalase activity assay was realized using the Amplitite™ Fluorimetric Catalase Assay Kit (AAT Bioquest Inc® CA, USA). The assay was performed according to manufacturer’s recommendation. Briefly, the samples (50 µl containing 0.04 µg of total proteins) were incubated with 50 µl of H2O2-containing assay buffer, at room temperature for 30 min. Then, the catalase assay mixture (containing Amplitite™ Red substrate and Horseradish peroxidase) was added and the incubation was pursued for another 30 min. Therefore, the reduction in fluorescence intensity is proportional to catalase activity. The fluorescence of the final mixture was then determined on a BioRad iMark™ absorbance microplate reader at 576 nm.

Growth Kinetics in Broth and Stress Survival Assays—Stationary-phase bacterial cultures of LVS/p6gro, LVSΔtpr1/p6gro and LVSΔtpr1/p6gro-tpr1 mutant strains were diluted to a final OD600 of 0.1 in Schaedler-K3 broth. Exponential-phase bacterial cultures were diluted to a final concentration of 108 bacteria/ml and subjected to the following stress conditions: 10% ethanol, 0.03% H2O2, pH 4.0, 50 °C or 0.05% SDS. The number of viable bacteria was determined by plating appropriate dilutions of bacterial cultures on Chocolate Polyvitex plates at the start of the experiment and after the indicated durations. For experiments at 50 °C, aliquots of 0.25 ml in a 1.5 ml tube were placed at 50 °C statically and at the indicated times, the tubes were placed on ice, serially diluted, and plated. For experiments with H2O2, pH 4.0 or 0.05% SDS, cultures (5 ml) were incubated at 37 °C with rotation (100 r.p.m.) and aliquots were removed at indicated times, serially diluted and plated immediately. Bacteria were stermatered after 72 h incubation at 37 °C. Experiments were repeated independently at least twice and data represent the average of all experiments.

Multiplication in Macrophages—Intracellular multiplication assays were performed essentially as previously described (6). THP1 (ATCC Number: TIB-202™) cells were propagated in RPMI containing 5% fetal calf serum. Cells were seeded at a concentration of ~2 × 105 cells per well in 12-well cell tissue plates and monolayers were used 48 h after seeding. THP-1 cells were differentiated by treatment, with 200 ng ml−1 phorbol myristate acetate (PMA). THP1 macrophage monolayers were incubated for 60 min at 37 °C with the bacterial suspensions (multiplicities of infection 100) to allow the bacteria to enter. After washing (time zero of the kinetic analysis), the cells were incubated in fresh culture medium containing gentamicin (10 µg ml−1) to kill extracellular bacteria. At several time-points, cells were washed three times in RPMI, macrophages were lysed by addition of water and the titer of viable bacteria released from the cells was determined by spreading preparations on Chocolate agar plates. For each strain and time in an experiment, the assay was performed in triplicate. Each experiment was independently repeated at least three times and the data presented originate from one typical experiment.

Mice Virulence Assay—All animal experiments were carried out in accordance to the European guidelines and following the recommendations of the INSERM guidelines for laboratory animal husbandry, LVS/p6gro, LVSΔtpr1/p6gro and LVSΔtpr1/p6gro-tpr1-FTL_0206 strains were grown in Schaedler-K3 containing kanamycin to exponential growth phase and diluted to the appropriate concentration, six to 8 weeks old female BALB/c mice (Janvier, Le Genest-St-Ise, France) were injected each day subcutaneously with kanamycin (50 µl of 12 mg ml−1 solution) 1 day before and every day during the infection. Mice were intraperitoneally inoculated with 200 µl of bacterial suspension (corresponding to ~250 CFU). The actual number of viable bacteria in the inoculum was determined by plating dilutions of the bacterial suspension on chocolate plates. After 4 days, the mice were sacrificed. Homogenized spleen and liver tissue from the five mice were diluted and spread onto chocolate agar plates supplemented with kanamycin and the number of viable bacteria per organ determined.

Identification of Proteins by Mass Spectrometry—Protein samples were run on SDS-PAGE gels and silver stained (20). Gel lanes were excised and subjected to tryptic digestion with sequencing grade modified Trypsin (Promega), as described previously (15). Nano-LC-MS/MS analysis of in-gel digested samples was performed on an
Ultimate 3000 Rapid Separation Liquid Chromatography (RSLC) system (Dionex) coupled to LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific). Extracted peptides were dissolved in 0.1% (v/v) trifluoroacetic acid, 10% acetonitrile, and preconcentrated on a 75 μm i.d. reversed-phase (RP) trapping column and separated with an aqueous-organic gradient (solution “A”: 0.1% (v/v) formic acid in 5% (v/v) acetonitrile; solution “B”: 0.085% (v/v) formic acid in 80% (v/v) acetonitrile; flow rate 400 nL/min) on a 75 μm × 15 cm, 2 μm, 100 Å, Dionex). Elution gradient settings for standard protein samples were: 3 min at 0% B, 10 min from 0% B to 10% B, 7 min from 10% B to 20% B, 7 min from 20% B to 40% B, 4 min from 40% B to 50% B, 0.5 min from 50% B to 90% B, 5 min at 90% B, 0.5 min from 90% B to 0% B, 5 min at 0%. MS instrument settings were as follows: spray voltage 1.8 kV; capillary temperature 250 °C; FT full MS target 1,000,000 (maximum injection time 100 ms). 2 FTMS full scan were averaged (resolution 30,000 for LTQ-Orbitrap velos; positive polarity; centroid data; scan range 400 to 2000 m/z) and the 20 most intense signals were subjected to MS/MS in the collision-induced dissociation (CID) cell (resolution 15,000; centroid data) with dynamic exclusion (repeat count 1; exclusion list size 50; repeat/exclusion duration 12 s; exclusion mass width ± 10 ppm), preview mode for FTMS master scans, charge state screening, monoisotopic precursor selection and charge state rejection (charge state 1 and 4+) enabled. Activation type was CID with default settings.

LC-MS/MS data were extracted and raw files from the analysis of bands from each independent experiment were merged. For data analysis, peak lists were generated by Proteome Discoverer v1.2 (Thermo Scientific) and searched against all the concatenated sequences of all the subspecies of *F. tularensis* (14,148 sequences; available online on [http://www.uniprot.org/taxonomy/?query=complete%3Ayes+content%3Afrancisella-tularensis+tularensis&sort=score]) from UniProtKB/Swiss-Prot complete proteome database (release 2011_01; 524,420 sequences) using the Mascot search engine (version 2.2.07; Matrix Science).

Default parameters used were: fixed modification (Carbamidomethyl (C)), and variable modification (Oxidation (M)) were allowed as well as one missed cleavage. Enzyme was trypsin, monoisotopic peptide mass tolerance was ± 5 ppm (after linear recalibration), fragment mass tolerance was ± 0.5 Da, false discovery rate was lower than 2%. Only proteins identified with at least two unique peptides and with the ion score higher than 25 were retained.

**RESULTS**

*In Silico Analyses*—We have recently identified a genetic locus, conserved among *Francisella* genomes, that comprises ten consecutive genes in the same orientation (*FTL_0200* to *FTL_0209*, in *F. tularensis* LVS). Deletion of gene *moxR* (*FTL_0200*) led to a mutant bacterium with increased vulnerability to various stress conditions and a growth defect in infected macrophages (6). Remarkably, the proximal part of the *moxR* operon, which is conserved in the genomes of several other pathogenic bacterial species, encodes proteins containing either tetratricopeptide repetition (TPR) or von Willebrand type A (VWA) motifs (Fig. 1). Confirming the peculiar characteristics of this genetic region, we identified only a limited number of proteins bearing either TPR or VWA motifs in the *F. tularensis* genomes currently available in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, us-
ing the Superfamily prediction program (available at http://supfam.cs.bris.ac.uk). Indeed, FTL_0201 (here designated VWA1), FTL_0203 and FTL_0204, were the only three proteins predicted to contain VWA-like motifs; and four proteins, including FTL_0205 (here designated TPR1), were predicted to contain TPR motifs with significant hits.

We identified a multiple 9 bp repeat in the middle part of gene tpr1 (FTL_0205), accounting for the presence of a NKD tripeptide repeat in the central part of the TPR1 protein (Fig. 1). The presence of 25 regions of 9 bp repeats (designated SSTR9 for short sequence tandem repeat with 9 nucleotides) had been previously reported in the genome of F. tularensis SCHU S4 (21). Twelve of these repeats were identified within predicted open reading frames, one of which corresponds to the ortholog of tpr1. Multiple amino acid sequence alignments reveal that the number of repetitions of the NKD motif varies among the different TPR1 protein homologs i.e. 32 repeats in F. tularensis subsp. novicida and 36 repeats in Francisella philomiragia ortholog (as compared with 16 repeats in TPR1 of LVS) (Fig. 1A). Hence, the protein TPR1 contains both a TPR motif in its proximal part and a NKD repeat region in its central part, suggesting unique structural properties for this protein (Fig. 1B).

In F. tularensis subsp. tularensis SCHU S4, a careful re-examination of the gene designated FTT_0294, which corresponds to the two distinct genes FTL_0204 and FTL_0205 in LVS, revealed it was erroneously annotated as a pseudogene in the KEGG database. In fact, the DNA sequence of FTT_0294 encompasses two consecutive coding sequences with a 10 bp overlap (supplemental Fig. S1A). The proximal part of FTT_0294 encodes a protein sharing 99.7% identity with FTL_0204 and the distal part of FTT_0294, a protein sharing 84.1% identity with FTL_0205 (TPR1) (supplemental Fig. S1B).

Identification of In Vivo Interaction Partners—Tandem affinity purification (TAP) was used to identify proteins interacting in vivo with proteins of the moxR locus, using a dual affinity tag coupled to mass spectrometry (8). We focused in the present work on MoxR, and on one protein for each type of protein–protein interaction motif. Our working hypothesis was that the interactomes of these baits would lead to the identification of other moxR-encoded proteins. VWA1 was chosen as a prototypic VWA-bearing motif protein because gene vwa1 was the only gene (of the three genes encoding VWA-bearing proteins), to be significantly up-regulated upon infection of macrophages (22). We thus considered that it was the most relevant candidate to identify interacting partners possibly involved in Francisella intracellular survival and virulence. TPR1 was chosen because it was the only protein of the moxR locus to bear a TPR motif and the only TPR protein encoded by the Francisella genome that also carried a NKD tripeptide repeat.

The three TAP-tagged proteins constructed were designated MoxR-TAP, VWA1-TAP and TPR1-TAP, respectively (see Experimental procedures for details). The corresponding recombinant genes were expressed under the control of the pgro promoter in the pFNLTP6pgro expression vector (Fig. 2A). The resulting plasmids were introduced in the F. tularensis LVS strain and expression of the tagged-proteins was verified by Western blot, using anti-tag antibody (15) on bacterial lysates. As shown in Fig. 2B, a band with the expected molecular weight (MW) was detected for the three recombinant proteins. An additional lower MW was also detected for MoxR-TAP, likely to correspond to a C-terminal proteolytic degradation product.

The three recombinant proteins were used individually as baits and their interacting LVS proteins were purified using a two-step elution procedure (15). In most cases, several bands were specifically eluted by this procedure (Fig. 2C). In each case, the MS-MS data obtained for each band were combined and analyzed to compact results in a single table for simplification.

Experiments were performed in biological replicates. One-dimensional SDS-PAGE protein profiles and MS-MS protein identifications obtained for each experiment were always compared with verify reproducibility. Results issued from triplicate experiments for MoxR-TAP and VWA1-TAP and duplicate experiments for TPR1-TAP are summarized in Tables IA–C. In each table, we chose as a threshold, the protein partners identified with at least two unique peptides and identified in at least two independent experiments.

Thirty-one different proteins interacting with MoxR-TAP were identified (Table IA), including MoxR-TAP itself. The most highly represented protein was FTL_1714 (or GroEL). The other proteins identified encompassed various predicted biological activities (ranging from ribosomal proteins to hypothetical proteins). Eight proteins appeared to interact with VWA1-TAP, including VWA1-TAP itself (Table IB). The two most highly represented proteins (peptide number >10) were FTL_1714 (GroEL) and FTL_1783 (or SucB), the E2 component of the 2-oxoglutarate dehydrogenase (OGDH) complex. Remarkably, TPR1 was also identified as a binding partner of VWA1-TAP. Finally, one hundred proteins interacting with TPR1-TAP were identified. These proteins belonged to various functional categories (Table IC). TPR1-TAP itself was among the most highly represented proteins. The other highly represented proteins (18 proteins with >10 unique peptides) included notably the E1 components of OGDH and pyruvate dehydrogenase (PDH). The E2 components of the two complexes were also detected (FTL_1783, OGDH E2; and FTL_0310, PDH E2) as well as the E3 component common to both complexes (FTL_0311, PDH/OGDH E3). Only one protein, FTL_0207, encoded by the moxR locus was identified among the interactants of TPR1-TAP. In addition, the two subunits of the acetyl-CoA carboxyl transferase (ACC-α and ACC-β) were identified (12 peptides each).

BACTH Analyses—The bacterial two-hybrids (BACTH) system was used to confirm several of the interacting partners
identified in the TAPtag approach. We focused in particular on the interactions of MoxR and TPR1 with the OGDH E2 subunit (FTL-1783 or SucB), as well as on the interactions of TPR1 with the three subunits of PDH (FTL_0309, FTL_0310, FTL_0311) and with the acetyl CoA carboxylase-
[260x139]subunit
[270x139]
(FTL_0295). The FTL proteins moieties were fused to the proximal portion (designated CyaA-T25, vectors pKT25 and pKNT25) or to the distal portion (designated CyaA-T18, vector pUT18C) of the catalytic domain of adenylate cyclase (Table II). Briefly, genes moxR and tpr1 were cloned into plasmid pKT25 (C-terminal fusions to CyaA-T25). The gene tpr1 was also cloned into plasmid pKNT25 (N-terminal fusion to CyaA-T25). The genes FTL-1783, FTL-0309, FTL-0310, FTL-0311, FTL-0295, were cloned into plasmid pUT18C encoding the distal domain of CyaA (C-terminal fusions to CyaA-T18). Each pair of recombinant bait (CyaA-T25, pKT derivatives)/pray (CyaA-T18, pUT derivatives) plasmids was introduced into the E. coli cya-strain BTH101. The interaction CyaA-T25 + CyaA-T18, reconstituting catalytic CyaA activity and leading to the production

**Fig. 2. Construction of MoxR, VWA1 and TPR1 tagged proteins.**

A, Constructions. Left panel, the 5.1 kb p6gro-derived recombinant plasmid carrying the fusion gene tpr1-Tap under the control of the pgro promoter. Right panel, schematic representation of the different parts the TPR1-TAP protein. The protein A and calmodulin binding (CBP) domains of the TAP sequence have been fused immediately downstream of the last residue of TPR1. B, Western-blot analyses. The recombinant proteins were expressed from p6gro-derived plasmids in LVS. The three proteins carry the TAPtag sequence at their C-terminal end. Western blot analysis was performed on whole cell extracts to verify production and stability of TAP-tagged proteins, using a polyclonal anti-TAPtag antibody. The three recombinant proteins were detected at the expected molecular weight (a, MoxR-TAP; b, VWA1-TAP; c, TPR1-TAP). The open arrow indicates the position of a probable degradation product of MoxR-TAP. Wild-type LVS strain was used as a negative control. C, TAP tag purification. TL, whole cell lysate; FT1, flow-through of the first purification column; FT2, flow-through of the second purification column; E2, final elution sample from the second column. The arrows indicate the protein band in which peptides corresponding to the bait were the most abundant. Molecular weight markers are indicated to the left of the figure (in kDa).
## Table I

| Accession UniProtKB | Gene name | FTL number | Protein description | Unique peptides |
|---------------------|-----------|------------|---------------------|-----------------|
| A. MoxR-TAP. Dieppedale et al. | | | | |
| P94798 | groEL | FTL_1714 | 60 kDa chaperonin** | 36 |
| Q2A5K6 | moxR | FTL_0200 | MoxR family ATPase** | 23 |
| Q9ZAW3 | ftsZ | FTL_1907 | Cell division protein FtsZ** | 12 |
| Q2A5L9 | pheC | FTL_0187 | Cyclohexadienyl dehydratase* | 8 |
| Q2A328 | dnaK | FTL_1191 | Chaperone protein DnaK* | 8 |
| Q2A1M4 | rplA | FTL_1747 | 50S ribosomal protein L1* | 7 |
| Q2A1Z7 | pckA | FTL_1616 | Phosphoenolpyruvate carboxykinase* | 7 |
| Q2A358 | iglD | FTL_0114/FTL_1160 | Intracellular growth locus protein D* | 7 |
| Q2A3U7 | pcoA | FTL_0885 | PhoH-like protein** | 6 |
| Q2A639 | dnaN | FTL_0002 | DNA polymerase III β subunit** | 5 |
| Q2A499 | rpsB | FTL_0702 | Hypothetical protein* | 5 |
| Q2A5G4 | rpsC | FTL_0242 | 30S ribosomal protein S3* | 4 |
| Q2A360 | iglB | FTL_0112/FTL_1158 | Intracellular growth locus protein B* | 4 |
| Q2A5E6 | rplC | FTL_0236 | 50S ribosomal protein L3* | 3 |
| Q2A5H0 | atpA | FTL_0261 | DNA-directed RNA polymerase 1 subunit* | 3 |
| Q2A4R4 | sucB | FTL_0891 | Hypothetical protein 3 | 2 |
| Q2A353 | mdh | FTL_0987 | Prolactin-like protein | 2 |
| Q2A51 | tsf | FTL_0125 | DNA-directed RNA polymerase α2 subunit* | 2 |
| Q2A3U2 | tig | FTL_0225 | Hypothetical protein | 2 |
| Q2A54 | tsf | FTL_0225 | DNA-directed RNA polymerase α2 subunit* | 2 |
| Q2A52 | tig | FTL_0225 | Hypothetical protein | 2 |
| Q2A1I0 | tuf | FTL_0261 | DNA-directed RNA polymerase α subunit* | 2 |
| Q2A1J4 | tuf | FTL_0261 | DNA-directed RNA polymerase α subunit* | 2 |
| Q2A368 | pyk | FTL_1148 | Glyceraldehyde-3-phosphate dehydrogenase* | 2 |
| Q2A368 | pyk | FTL_1148 | Glyceraldehyde-3-phosphate dehydrogenase* | 2 |

18. VWA1-TAP. Dieppedale et al. | | | | |
| P94798 | groEL | FTL_1714 | 60 kDa chaperonin** | 43 |
| Q2A1J4 | sucB | FTL_1782 | Oxoglutarate dehydrogenase E2 component* | 14 |
| Q2A328 | dnaK | FTL_1191 | Chaperone protein DnaK* | 8 |
| Q2A5E0 | htpG | FTL_0267 | Chaperone protein HtpG* | 4 |
| Q2A5K5 | vwa1 | FTL_0201 | Hypothetical protein | 3 |
| Q2A1M0 | tuf | FTL_0205 | Elongation factor Tu* | 3 |
| Q2A5K1 | tpr1 | FTL_1148 | Tetrameric peptide repeat* | 2 |
| Q2A368 | pyk | FTL_1148 | Pyruvate kinase* | 2 |

1C. TPR1-TAP. Dieppedale et al. | | | | |
| Q2A328 | dnaK | FTL_1191 | Chaperone protein DnaK* | 55 |
| Q2A5E0 | htpG | FTL_0267 | Chaperone protein HtpG* | 34 |
| P94798 | groEL | FTL_1714 | 60 kDa chaperonin* | 21 |
| Q2A5A0 | aceE | FTL_0309 | Pyruvate dehydrogenase E1 component | 21 |
| Q2A5K1 | tpr1 | FTL_0205 | Tetrameric peptide repeat* | 19 |
| Q2A4Q0 | gyrA | FTL_0533 | DNA gyrase, subunit A | 17 |
| Q2A5V6 | clpB | FTL_0094 | AAA superfamily ATPase ClpB | 15 |
| Q2A1M4 | rplA | FTL_1747 | 50S ribosomal protein L1* | 15 |
| Q2A51 | tuf | FTL_0224 | 30S ribosomal protein S2* | 15 |
| Q2A1G8 | tuf | FTL_1809 | Translation initiation factor IF-2* | 14 |
| Q2A1J3 | sucA | FTL_1782 | Oxoglutarate dehydrogenase E1 component | 14 |
| Q2A432 | tuf | FTL_0785 | GTP-binding protein | 13 |
| Q2A52 | tuf | FTL_0785 | GTP-binding protein | 13 |
| Q2A5B4 | accA | FTL_0295 | Acetyl-CoA carboxylase α subunit | 12 |
| Q2A39 | tuf | FTL_0894 | Acetyl-CoA carboxylase β subunit* | 12 |
| Q2A1M8 | tuf | FTL_1743 | DNA-directed RNA polymerase β subunit* | 12 |
| Q2A1K3 | tuf | FTL_1772 | Acetolactate hydratase | 11 |
| Accession | UniProtKB | Gene name | FTL number | Protein description | Unique peptides |
|-----------|------------|-----------|------------|---------------------|-----------------|
| Q2ASG4    | rpsC       | FTL_0242  | 30S ribosomal protein S3* | 11               |
| Q2A360    | igIB       | FTL_0112/FTL1158 | Intracellular growth locus protein B* | 10               |
| Q2A180    | rpsA       | FTL_1912  | 30S ribosomal protein S1* | 9                |
| Q2A298    | katG       | FTL_1504  | Catalase-peroxidase* | 9                |
| Q2A2S5    | dnaJ       | FTL_1306  | Hypothetical protein | 9                |
| Q2ASC7    |            |           |            |                    |
| Q2A1J4    | sucB       | FTL_1783  | Oxoglutarate dehydrogenase E2 component* | 9               |
| Q2A632    | ompH       | FTL_0009  | Outer membrane protein* | 9                |
| Q2A5E6    | rpsA       | FTL_0260  | 30S ribosomal protein S4* | 9                |
| Q2A298    | katG       | FTL_1504  | Catalase-peroxidase* | 9                |
| Q2A2S5    | dnaJ       | FTL_0281  | Hypothetical protein | 9                |
| Q2A598    | lpd        | FTL_0311  | Dihydrolipoyl dehydrogenase* | 8               |
| Q2A599    | aceF       | FTL_0310  | Pyruvate dehydrogenase E2 component* | 5               |
| Q2A110    | atpA       | FTL_1797  | FOF1 ATP synthase subunit | 5                |
| Q2A2J1    | inFC       | FTL_1406  | Translation initiation factor IF-3 | 5                |
| Q2A2H7    |            | FTL_1420  | Carbohydrate/purine kinase PkIB family | 5                |
| Q2A5F5    | rplF       | FTL_0251  | 50S ribosomal protein L5 | 5                |
| Q2A193    | glnA       | FTL_1899  | Glutamine synthetase* | 4                |
| Q2A4K8    | fabD/acbP  | FTL_0584  | Hydroxacyl-CoA dehydrogenase/acyl-CoA-binding protein | 4               |
| Q2A377    | fabG       | FTL_1139  | 3-oxoacyl-(Acyl-carrier) reductase* | 4               |
| Q2A2C4    |            | FTL_1477  | Thiamine pyrophosphokinase | 4                |
| Q2A263    | mutM       | FTL_1543  | Formamidopyrimidine-DNA glycosylase | 4                |
| Q2A2T8    |            | FTL_1293  | Hypothetical protein | 4                |
| Q2A502    | parB       | FTL_0426  | Chromosome partition protein B | 4                |
| Q2A5X6    | lepA       | FTL_0071  | GTP-binding protein LepA | 4                |
| Q2A269    | pnp        | FTL_1537  | Polynucleotide phosphorylase/polyadenylase* | 4               |
| Q2A36    |            | FTL_1015  | AhpC/TSA family protein- Peroxiredoxin* | 4               |
| Q2A4M0    |            | FTL_0572  | Hypothetical protein* | 4                |
| Q2A17    | rpoB       | FTL_1744  | DNA-directed RNA polymerase β subunit | 4                |
| Q2A2E3    | secA       | FTL_1458  | Protein translocase subunit SecA | 4                |
| Q2A303    |            | FTL_1216  | Hypothetical protein | 4                |
| Q2A5S1    | ppdK       | FTL_0132  | Pyruvate phosphate dikinase | 4                |
| Q2A259    | gyrB       | FTL_1547  | DNA gyrase β subunit | 3                |
| Q2A2K5    | deaD       | FTL_1392  | cold-shock DEAD-box protein A | 3                |
| Q2A3E5    | dacD       | FTL_1060  | d-alanyl-d-alanine carboxypeptidase* | 3               |
| Q2A358    | igID       | FTL_0114/FTL_1160 | Intracellular growth locus protein D | 3               |
| Q2A346    | pdpA       | FTL_0162/FTL_1172 | Pathogenicity determinant protein PdpA | 3               |
| Q2A1F6    | nuoG       | FTL_1824  | NADH dehydrogenase subunit G | 3                |
| Q2A4R4    | minD       | FTL_0519  | Septum site-determining protein MinD | 3                |
| Q2A3U7    | phoH       | FTL_0885  | PhoH-like protein | 3                |
| Q2A366    | pdpC       | FTL_0116/FTL_1162 | Pathogenicity determinant protein PdpC | 3               |
| Q2A4B2    | arac       | FTL_0689  | Transcriptional regulator AraC family | 3                |
| Q2A5H2    | fusA       | FTL_0234  | Elongation factor G | 3                |
| Q2A329    | grpE       | FTL_1190  | Heat shock protein GrpE* | 3               |
| Q2A374    | plsX       | FTL_1142  | Glycerol-3-phosphate acyltransferase PIsX* | 3               |
| Q2A5P0    | usp        | FTL_0166  | Universal stress protein* | 3               |
| Q2A415    | putA       | FTL_0805  | Bifunctional proline dehydrogenase | 3                |
| Q2A580    | tolB       | FTL_0334  | Protein TolB | 3                |
| Q2A639    | dnaN       | FTL_0002  | DNA polymerase III β subunit | 2                |
| Q2A211    | hemB       | FTL_1602  | Delta-aminolevulinic acid dehydratase | 2               |
of cAMP, was monitored by the hydrolysis of the lactose analog X-Gal on LB- X-Gal plates. As summarized in Table II, MoxR-CyaA-T25 and FTL_1783-CyaA-T18 recombinant proteins interacted together to produce cAMP. The TPR1-CyaA-T25 N-terminal fusion interacted with all the protein partners tested whereas the CyaA-T25-TPR1 C-terminal fusion interacted only with 3/5 of them. Altogether, this assay confirmed the interactions of MoxR with the E2 subunit of the OGDH complex; and of TPR1 with the OGDH E2 subunit, the PDH complex as well as with the ACC α subunit.

**Construction and Characterization of a Δtpr1 Chromosomal Deletion**—To further characterize the role of the protein TPR1 in *Francisella* pathogenesis, we constructed a clean chromosomal deletion of gene *tpr1* by allelic replacement, and a complemented strain by introducing a plasmid-born wild-type *tpr1* allele in LVS. Expression of gene *tpr1* was abolished in the deletion strain and introduction of a plasmid-born wild-type *tpr1* allele led to high level expression of the *tpr1* gene (supplemental Fig. S2). We also monitored expression of the gene FTL_0206 lying immediately downstream of *tpr1* (Fig. 1), in these strains. An ~2-fold reduction in FTL_0206 gene expression was recorded in the Δtpr1 mutant background (supplemental Fig. S2), reflecting a polar effect of the clean deletion. Reduced expression of FTL_0206 was still observed in the *tpr1*-complemented strain. We therefore constructed a new complemented strain by introducing in the Δtpr1 mutant a plasmid expressing both *tpr1* and FTL_0206 genes. In this new complemented strain, expression of both genes *tpr1* and FTL_0206 was fully restored (supplemental Fig. S2). The characteristics of the wild-type, Δtpr1 mutant and complemented strains were followed in different stress conditions as well as in human macrophages and *in vivo*.

**Sensitivity to Oxidative, pH and Other Stresses**—Upon *Francisella* entry into cells, the phagosomal compartment transiently acidifies and the activation of NADPH oxidase leads to the production of noxious oxygen reactive species (23, 24). Thus, we first tested the survival of parental LVS, LVS/Δtpr1 mutant and complemented strains, under oxidative stress conditions.

For this, bacteria were exposed to 0.03% H2O2 (~10 mM) (Fig. 3). The Δtpr1 deletion mutant strain (LVS/Δtpr1/p6gro) appeared to be extremely sensitive to oxidative stress as compared with the wild-type strain (LVS/p6gro) Indeed, it showed a 25-fold decrease in the number of viable bacteria already after 10 min, and an ~50,000-fold decrease after 30 min of exposure to H2O2. Remarkably, the two complemented
strains, harboring either a plasmid-born copy of gene tpr1 alone (plasmid p6gro-tpr1) or of genes tpr1 and FTL_0206 (p6gro+tpr1 or p6gro-tpr1-FTL_0206) showed wild-type bacterial viability (Fig. 3). This assay confirmed that protein TPR1 specifically contributes to the adaptation of F. tularensis to oxidative stress. Notably, we also evaluated the capacity of the protein TPR1-TAP to complement the Δtpr1 deletion mutant. This strain (LVSΔtpr1/p6gro-tpr1-TAP) showed resistance to oxidative stress similar to LVS and complemented strains, indicating a normal activity of the TPR1-TAP protein despite the presence of the C-terminal tag.

We next evaluated the ability of the LVSΔtpr1 mutant to endure acid stress, by incubating the LVS, LVSΔtpr1 and of the complemented strain (LVSΔtpr1/p6gro-tpr1-FTL_0206), in normal growth media adjusted to pH 4.0 (supplemental Fig. S3A). Both LVS and LVSΔtpr1 were sensitive to low pH, but the LVSΔtpr1 mutant strain showed a 2,300-fold increase of mortality after 4 h. The complemented strain exhibited viability at the same level as the wild-type parent.

We also evaluated the impact of the Δtpr1 deletion on bacteria subjected to heat, ethanol and SDS stresses. When subjected to high temperature (50 °C) (supplemental Fig. S3B), the number of viable bacteria of the LVSΔtpr1 mutant was 14-fold lower than that of the wild type strain after 1 h. In media containing 0.05% SDS (supplemental Fig. S3C), the number of mutant bacteria was 350-fold lower than of LVS bacteria after 4 h and the mutant resisted 15-fold less than LVS after 2 h and in media containing a high concentration of ethanol (10%) (supplemental Fig. S3D). In all four assays, the complemented strain (LVSΔtpr1/p6gro-tpr1-FTL_0206) exhibited viability at the same level as the wild-type parent.

Thus, the Δtpr1 mutant appeared to be more sensitive than the wild-type strain to all the stresses tested, a characteristic resembling that previously observed with the ΔmoxR mutant (6). Remarkably, the ΔmoxR and Δtpr1 mutants were both more sensitive to oxidative stress than to any other stress. Altogether, these data indicate that the proteins MoxR and TPR1 contribute to the adaptation of F. tularensis to a variety of stressful conditions and are particularly important for oxidative stress defense.

Intracellular Survival and Multiplication—We then examined the ability of the wild-type, Δtpr1 mutant, and complemented strains, to multiply in human macrophages (THP-1 cell line) over a 48 h period. The Δtpr1 mutant (LVSΔtpr1/p6gro) showed an ~10-fold reduction of intracellular bacteria after 24 h and 48 h, as compared with the parental strain (LVS/p6gro) (Fig. 4A). Introduction of the complementing plasmid p6gro-tpr1 restored partially bacterial multiplication in THP-1 cells. Indeed, after 24 h and 48 h of infection, multiplication of the complemented strain was improved as

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**Fig. 3. Sensitivity to oxidative stress of LVS, LVSΔtpr1 and complemented strains.** Exponential-phase bacteria were diluted to a final concentration of 10⁶ bacteria/ml in fresh Schaedler-K3 broth and subjected to oxidative stresses (10 mM H₂O₂). The bacteria were plated on chocolate agar plates at different times, and viable bacteria were monitored 3 days after. Data are the average CFU/ml for three independent experiments for each condition. Results are shown as the averages of log₁₀ (CFU/ml) ± standard deviations. ** p < 0.01 (as determined by Student’s t test).

**Fig. 4. In vitro and vivo properties of LVSΔtpr1.** A. Intracellular replication of LVS, LVSΔtpr1 and the complemented strains was monitored over a 48 h period in THP-1 human macrophages. Results are shown as the averages of log₁₀ (CFU/ml) ± standard deviations. ** p < 0.01 (as determined by Student’s t test). B. Bacterial burden in spleen and livers of mice infected with 250 CFU of wild-type LVS (LVS/p6gro, black diamonds), Δtpr1 mutant (LVSΔtpr1/p6gro, open triangles) or complemented strain (LVSΔtpr1/p6gro-tpr1-FTL_0206, open diamonds). Results are shown as log₁₀ CFU per organ ± standard deviations. Liver and spleen data at day 4, for LVS and LVSΔtpr1 infections, represent five mice. * p < 0.05; ** p < 0.01; *** p < 0.001 (as determined by Student’s t test).
compared with the deletion strain (−fivefold higher) but was still lower than that of the parental strain. The complemented strain expressing both tpr1 and FTL_0206 genes (LVS\(\Delta\)tpr1\(\Delta\)p6gro-tpr1-FTL0206) showed wild-type intracellular survival and multiplication in macrophages (Fig. 4A), indicating that both tpr1 and FTL_0206 genes contribute to LVS intracellular multiplication.

**In Vivo Properties**—We examined the virulence of wild-type LVS (LVS/p6gro), \(\Delta\)tpr1 mutant (LVS\(\Delta\)tpr1/p6gro) and complemented strain (LVS\(\Delta\)tpr1/p6gro-tpr1-FTL_0206), in mice, by following the kinetics of bacterial multiplication in the target organs (spleen and liver) of infected animals (Fig. 4B). Groups of five BALB/c mice were infected with 250 bacteria by the intra-peritoneal route and mice were sacrificed at day 4 after the inoculation. At day 4, multiplication in the \(\Delta\)tpr1 mutant strain significant decreased as compared with the wild-type strain in both organs (25-fold and 63-fold, in the spleen and in the liver, respectively). Remarkably, complementation with plasmid p6gro-tpr1-FTL0206 restored full virulence. Indeed, similar counts were recorded in the spleens of animals infected with the wild-type and complemented strains (log\(_{10}\) 6.2 versus log\(_{10}\) 6.38, respectively). The counts recorded in the livers were even 10-fold higher with the complemented strain than with the wild-type strain (Fig. 4B). These results thus confirm our earlier preliminary observations (6) on the participation of genes tpr1 and FTL_0206 in F. tularensis virulence.

**MoxR and TPR1 Modulate OGDH and PDH Enzymatic Activities**—As mentioned above, all the subunits of the 2-oxoglutarate dehydrogenase (OGDH) and pyruvate dehydrogenase (PDH) complexes interacted with TPR1-TAP. The TPR-bearing motif protein also interacted with the two subunits of Acetyl-CoA carboxylase (ACC). This observation led us to test the impact of moxR or tpr1 inactivation on the activity of these three enzymatic complexes. The OGDH complex is a key enzyme in the TCA cycle which catalyzes the conversion of 2-oxoglutarate to succinyl-CoA, NADH and CO\(_2\) (25). The PDH complex, an enzyme linking the glycolysis pathway and the TCA cycle, catalyzes the conversion of pyruvate in acetyl-CoA, NADH and CO\(_2\) (25, 26). Acetyl-CoA carboxylase (ACC) is a biotin-dependent multi-subunit enzyme (19) that catalyzes the irreversible carboxylation of acetyl-CoA to produce malonyl-CoA. This irreversible reaction is involved in fatty acid synthesis.

OGDH activity was assayed on bacterial lysates by measuring the reduction of NAD\(^+\) at 340 nm in the presence of 16 mM oxoglutarate. PDH activity was assayed in the same conditions in the presence of 16 mM pyruvate. ACC activity was assayed on bacterial lysates by measuring the amount of coenzyme A formed at 412 nm, in the presence of malonyl-CoA (see Experimental procedures).

The protein TPR1 also interacted with the catalase KatG, responsible for the detoxification of bactericidal compounds such as H\(_2\)O\(_2\). The fact that KatG has been shown previously to participate to F. tularensis oxidative stress response (4) and references therein), prompted us to monitor the catalase enzymatic activity in wild-type, \(\Delta\)tpr1 and complemented strains. Catalase activity was assayed on bacterial lysates by measuring the conversion of H\(_2\)O\(_2\) into H\(_2\)O and O\(_2\), using the Amplex\textsuperscript{TM} Fluorimetric Catalase Assay Kit (see Experimental procedures).

As shown in Fig. 5, PDH and OGDH activities dropped down to 30\% of the wild-type activity in the lysate from the LVS\(\Delta\)moxR mutant strain. Both enzymatic activities were also significantly reduced in the lysate from LVS\(\Delta\)tpr1 (18 and 29\% reduction of activity for PDH and OGDH, respectively). We monitored expression of genes FTL_0309 and FTL_1784 (encoding PDH- and OGDH- E1 subunits, respectively) by qRT-PCR, in LVS and in the LVS\(\Delta\)tpr1, strains (supplemental Fig. S4). Transcription of these two genes was unaffected in the \(\Delta\)tpr1 mutant, strongly suggesting that the decrease in PDH and OGDH activities in this strain was not due to reduced protein expression.

KatG activity was also moderately but significantly reduced in the \(\Delta\)tpr1 mutant strain (17\% reduction of activity) and wild-type activity was fully restored in the complemented strain (Fig. 5F). The background activity recorded in the \(\Delta\)tpr1 strain is likely to be due to the remaining H\(_2\)O\(_2\) detoxifying activity of glutathione peroxidase and alkyl-hydroperoxide reductase. This assay confirmed, thus, the functional relevance of the interaction between the proteins TPR1 and KatG. Of note, inactivation of katG, in both F. tularensis subsp. tularensis strain SCHU S4 or F. tularensis subsp. holarctica strain LVS, resulted in enhanced susceptibility to H\(_2\)O\(_2\) in vitro. The oxidative stress defect of the LVS\(\Delta\)tpr1 mutant (see above) might thus be accounted by, at least in part, to an effect on this protein.

In contrast, ACC activity was not significantly affected in any of the two mutants as compared with LVS. Hence, the interaction of TPR1 with this enzyme is not critical for its activity, at least in the conditions tested.

**DISCUSSION**

Using in vivo tandem affinity purification, combined with high-resolution mass spectrometry, we identified the interactomes of MoxR, VWA1 and TPR1, three proteins encoded by the moxR locus of F. tularensis LVS. The interacting proteins belonged to various functional categories, notably including key metabolic enzymes such as PDH and OGDH. We show here that inactivation of tpr1 impairs bacterial virulence and affects bacterial stress defense. Remarkably, in both moxR and tpr1 mutants, the activities of PDH and OGDH were altered, suggesting a possible link between stress defense and the tricarboxylic acid cycle in F. tularensis virulence.

**The Interactomes Show Partial Overlap**—We successfully identified some interactions between moxR-encoded proteins (VWA1 with TPR1, and TPR1 with FTL_0207). However, we did not identify the reciprocal interactions (e.g. VWA1 was not identified in the TPR1-TAP interactome), possibly due to hin-
drance caused by the presence of the C-terminal Tag. Further biochemical and structural studies will be required to characterize the nature of the oligomeric structures comprising these proteins.

In addition, to the moxR-encoded proteins, the three interactomes comprised a subset of common proteins. Remarkably, most of the proteins interacting with MoxR-TAP also interacted with either one or both of the two other TAP-tagged proteins. For example, two-third of the proteins interacting with MoxR-TAP (19/31) also interacted with TPR1-TAP. These included: 1) the general chaperones GroEL and DnaK; 2) trigger factor (Tig) and EF-Tu, involved in polypeptide chain elongation; 3) FtsZ and MinD, involved in cell division; 4) the pyruvate kinase (Pyk) and the OGDH E2 component, involved in metabolism; and v) the *Francisella* pathogenicity island proteins IgIB and IgID. Similarly, all the proteins interacting with VWA1-TAP also interacted with proteins from the two other TAP-tagged interactomes. The proteins GroEL and DnaK constituted the most abundant peptides in most interactomes. It cannot be excluded that they correspond to non-specific protein–protein interactions because these two proteins are highly abundant in *F. tularensis* (27, 28).

The protein TPR1-TAP interacted with numerous proteins, including several enzymes or enzymatic complexes that were not detected in the other two interactomes.

### Enzymatic assays

The assays were performed on total protein lysates. Panels A, B, C, compared the activities in wild-type LVS to those in ΔmoxR and Δtpr1 mutant strains. Panels D, E, F, compared the activities in wild-type LVS to those in Δtpr1 and complemented strains (LVSΔtpr1/p6gro-tpr1 and LVSΔtpr1/p6gro-tpr1-FTL_0206). (A, D) Pyruvate dehydrogenase assays were realized at 340 nm, over a reaction time of 10 min. (B, E) Oxoglutarate dehydrogenase assays were realized at 340 nm, over a reaction time of 5 min. (C) Acetyl-CoA carboxylase assay was realized at 412 nm over a reaction time of 5 min. Values were collected every minute. Each assay was performed on three independent protein lysates and each experiment was repeated twice. The average of absorbance ± standard deviations, recorded for the wild-type LVS and mutant strains at the end of the reaction were significantly different (PDH, \( p < 0.05 \); OGDH, \( p < 0.01 \), as determined by Student’s t test). Ordinate, variation of absorbance. Abscissa, time (in min) of the reaction. F. Catalase activity assay was realized at 576 nm, using the Amplite™ Fluorimetric Catalase Assay Kit. The assay was performed according to manufacturer’s recommendation. Each assay was performed on three independent protein lysates and each experiment was repeated twice. The average of catalase activity (in unit/mg of total proteins) ± standard deviations, recorded for the wild-type LVS and mutant strains at the end of the reaction were significantly different (\( p < 0.01 \), as determined by Student’s t test). Ordinate, catalase activity.

**Fig. 5.** Enzymatic assays. The assays were performed on total protein lysates. Panels A, B, C, compared the activities in wild-type LVS to those in ΔmoxR and Δtpr1 mutant strains. Panels D, E, F, compared the activities in wild-type LVS to those in Δtpr1 and complemented strains (LVSΔtpr1/p6gro-tpr1 and LVSΔtpr1/p6gro-tpr1-FTL_0206). (A, D) Pyruvate dehydrogenase assays were realized at 340 nm, over a reaction time of 10 min. (B, E) Oxoglutarate dehydrogenase assays were realized at 340 nm, over a reaction time of 5 min. (C) Acetyl-CoA carboxylase assay was realized at 412 nm over a reaction time of 5 min. Values were collected every minute. Each assay was performed on three independent protein lysates and each experiment was repeated twice. The average of absorbance ± standard deviations, recorded for the wild-type LVS and mutant strains at the end of the reaction were significantly different (PDH, \( p < 0.05 \); OGDH, \( p < 0.01 \), as determined by Student’s t test). Ordinate, variation of absorbance. Abscissa, time (in min) of the reaction. F. Catalase activity assay was realized at 576 nm, using the Amplite™ Fluorimetric Catalase Assay Kit. The assay was performed according to manufacturer’s recommendation. Each assay was performed on three independent protein lysates and each experiment was repeated twice. The average of catalase activity (in unit/mg of total proteins) ± standard deviations, recorded for the wild-type LVS and mutant strains at the end of the reaction were significantly different (\( p < 0.01 \), as determined by Student’s t test). Ordinate, catalase activity.
acetyl-CoA carboxylase (AccA, AccD), and KatG. Among these only the OGDH E2 subunit (SucB) belonged to another interactome (VWA1). This may reflect peculiar biochemical and/or structural properties of TPR1, favoring protein–protein interactions. The contribution of the other moxR-encoded proteins to these other enzymatic activities, if needed, might not require direct binding. Using the same threshold of 10 peptides, the TPR1 interactome shares eight proteins with the MoxR interactome and only three proteins with the VWA1 interactome (Fig. 6). The chaperones DnaK and GroEL are the only two proteins identified in the three interactomes.

Stress Response, the TCA Cycle and Virulence—Inactivation of either moxR or tpr1 impaired bacterial stress defenses and also significantly altered PDH and OGDH activities. OGDH is an enzymatic complex composed of multiple copies of three different subunits: the 2-oxoglutarate dehydrogenase (E1, FTL_1784), the dihydrolipoamide succinyltransferase (E2, FTL_1783), and the lipoamide dehydrogenase (lpd or E3, FTL_0311) that is shared with pyruvate dehydrogenase (supplemental Fig. S4). The OGDH complex, which constitutes the primary site of control of the metabolic flux through the TCA cycle, has been also shown to be involved in the protection of the bacterium against reactive nitrogen intermediates and oxidative stress generated by the host immune system (see for a review (29)). The PDH enzymatic complex is composed of three components: the pyruvate dehydrogenase (E1 component, FTL_0309), the dihydrolipoamide transacetylase (E2 component, FTL_0310) and the dihydrolipoamide dehydrogenase (E3 component, FTL_0311). In both complexes the E2 component forms a multimeric core, which binds the peripheral E1 and E3 subunits. The substrate is decarboxylated by the E1 component dependant of thiamine pyrophosphate. Then, it is oxidized and transiently acetylated by E2 component.

In F. tularensis, the genes encoding the E1 and E2 subunits of OGDH complex are predicted to be essential genes (30) which probably accounts for the fact that they have not been identified in earlier in vitro and in vivo genetic screens as potential virulence factors. In contrast, the genes encoding the E1 and E2 subunits of PDH, as well as that encoding the shared E3 subunit are not essential. Indeed, the gene aceE, encoding PDH E1 subunit in F. novicida (FTN_1494), was identified in a recent screen as important for intracellular survival (31). Inactivation of the lpd gene (encoding the E3 subunit) was also found to affect the virulence of F. tularensis LVS (32). Interestingly, in Mycobacterium tuberculosis, the Lpd protein is part of an antioxidant defense system in addition to its role in intermediary metabolism (33). Hence, it is also possible that Lpd participates in defense against oxidative stress in F. tularensis. Of note, in S. typhimurium, a
ΔsucAB mutant (lacking OGDH E1 and E2, and thus unable to make succinyl-CoA) was shown to be avirulent in the mouse model (34). Interestingly, Richardson et al. recently showed that S. typhimurium became auxotrophic for methionine and lysine under nitrosative stress, because of reduced succinyl-CoA availability (35).

Our work suggested possible connections between stress response and metabolism in pathogenic F. tularensis. The proteins MoxR, VWA1, TPR1, and possibly other moxR-encoded proteins, might assist multiple enzymatic activities, including the tricarboxylic acid cycle-related PDH and OGDH. Further work will be required to make precise the molecular mechanisms involved in these interactions and their importance for intracellular survival and multiplication of the bacterium. More generally, the fact that similar moxR-like loci exists in several other intracellular bacteria, such as for example Legionella species, suggest functions carried by moxR-encoded proteins might be shared by other pathogenic organisms.

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** The authors contributed equally to this work.

To whom correspondence should be addressed: Bâtiment Le riche. 96 rue Didot 75993 Paris Cedex 14 France. Tel.: 0033-1-72606511; E-mail: alain.charbit@inserm.fr.

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