The metabolic enzyme fructose-1,6-bisphosphate aldolase acts as a transcriptional regulator in pathogenic *Francisella*

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The enzyme fructose-bisphosphate aldolase occupies a central position in glycolysis and gluconeogenesis pathways. Beyond its housekeeping role in metabolism, fructose-bisphosphate aldolase has been involved in additional functions and is considered as a potential target for drug development against pathogenic bacteria. Here, we address the role of fructose-bisphosphate aldolase in the bacterial pathogen *Francisella novicida*. We demonstrate that fructose-bisphosphate aldolase is important for bacterial multiplication in macrophages in the presence of gluconeogenic substrates. In addition, we unravel a direct role of this metabolic enzyme in transcription regulation of genes *katG* and *rpoA*, encoding catalase and an RNA polymerase subunit, respectively. We propose a model in which fructose-bisphosphate aldolase participates in the control of host redox homeostasis and the inflammatory immune response.
Francisella tularensis is the causative agent of the zoonotic disease tularemia. This Gram-negative facultative intracellular pathogen can infect humans by different means including direct contact with sick animals, inhalation, insect bites, or ingestion of contaminated water or food. F. tularensis is able to infect numerous cell types, including dendritic cells, neutrophils, macrophages as well as hepatocytes, or endothelial cells but is thought to replicate in vivo mainly in macrophages. Four major subspecies of F. tularensis are currently listed: subsp tularensis (also designated Type A), subsp holarctica (also designated Type B), and F. tularensis subsp novicida. These subspecies differ in virulence and geographical origin but all cause a fulminant disease in mice that is similar to tularemia in humans. Although F. novicida is rarely pathogenic in humans, it is highly infectious in mice and its genome shares a high degree of nucleotide sequence conservation with the human pathogenic species. F. novicida is thus widely used as a model to study highly virulent subspecies.

Francisella virulence is tightly linked to its capacity to multiply in the cytosolic compartment of infected cells, and in particular of macrophages in vivo. The ability of Francisella to replicate within macrophages necessitates the coordinate control of three master transcription regulators, called MglA, SspA, and Pigg, which integrate the nutritional status of the pathogen to virulence gene expression in the host. Francisella belongs to the limited group of intracellular bacteria, notably Listeria monocytogenes and Shigella flexneri, that can gain access to and proliferate within the host cell cytosol. Cytosolic bacterial multiplication often requires the utilization of multiple host-derived nutrients and hexoses such as glucose are generally the preferred carbon and energy sources. In mammalian cells, glycolysis and the oxidative branch of the pentose-phosphate pathway occur in the cytosol, whereas the tricarboxylic acid (TCA) cycle, glutaminolysis, and β-oxidation take place in the mitochondria. In contrast, the glycolytic reactions (gluconeogenesis and amino acid, nucleotide, and fatty acids biosynthesis) occur mainly in the cytosol. Hence, the bacterial enzymes responsible for glucose metabolism (catabolism and anabolism) are likely to play a key role in intracellular bacterial adaptation.

In the present study, we decided to address the role of the unique class II fructose-1,6-bisphosphate aldolase (FBA) of Francisella, a ubiquitous metabolic enzyme occupying a central position in glycolysis and gluconeogenesis pathways. Remarkably, FBA has been recently reported to play a role in the pathogenesis of two important human pathogens, highlighting the importance of metabolism in pathogenesis. In M. tuberculosis, FBA was shown to be required for growth in the acute phase and for survival in the chronic phase of mouse infections. FBA was also shown to be essential for replication and virulence of the obligate intracellular parasite Toxoplasma gondii. Two different classes of FBAs, with different catalytic mechanisms, have been described according to their amino acid sequences and designated Class I- and Class-II FBAs, respectively. These aldolases have also been implicated in many moonlighting or non-catalytic functions, based upon their binding affinity for multiple other proteins, in both prokaryotic and eukaryotic organisms. Class I FBAs are usually found in higher eukaryotic organisms (animals and plants). They utilize an active site lysine residue to stabilize a reaction intermediate via Schiff-base formation. Class I FBAs have been shown to interact with proteins displaying different functions predominantly involved in cellular structure, including notably F-actin, WASP, phospholipase D, and V-ATPase. Class II FBAs are commonly found in bacteria, archaea and lower eukaryotes, including fungi and some green algae. Some bacterial species, including Escherichia coli, have been reported to express both types of the enzyme. Class II FBAs have been further subdivided into type A and type B, on the basis of the amino acid sequence. Of note, type A enzymes have been found mostly involved in glycolysis and gluconeogenesis, while diverse metabolic roles and substrate specificities have been reported for type B aldolases.

Several attempts to disrupt the Class II fructose biphosphate aldolase genes from different bacterial species, including Escherichia coli, Bacillus subtilis, and Pseudomonas aeruginosa, have been unsuccessful, thereby suggesting that the Class II FBP aldolase is essential for the viability of these organisms. In M. tuberculosis, FBA was indeed demonstrated to be essential.

Here we show that the gene fba is dispensable for Francisella survival and growth under defined conditions, and appears to play a regulatory role in pathogenesis. This enzyme appears to lie at the crossroad between carbon metabolism and the control of host redox homeostasis.

**Results**

**Metabolomics reveal high glucose consumption in infected BMMs.** To assess the impact of bacterial infection and multiplication on the metabolism of infected macrophages, we first analyzed the metabolome of mouse bone marrow-derived macrophages (BMMs) and compared the concentration of a series of metabolites recorded in non-infected (NI) cells to those recorded in infected cells. We used for infection, either wild-type (WT) F. novicida (WT) or a F. novicida mutant with a deletion of the fba gene. To assess the impact on the metabolism of infected macrophages, we used for infection, either wild-type (WT) F. novicida (WT) or a F. novicida mutant with a deletion of the fba gene.

The quantitative metabolomics analysis of intracellular metabolites was performed by IC-MS/MS. This approach allowed us to quantify central and intermediary metabolites from a number of metabolic pathways occurring in BMMs (i.e., glycolysis, gluconeogenesis, pentose-phosphate pathway, TCA cycle, nucleotides biosynthesis, and activated sugars biosynthesis) where F. novicida was unable to escape from phagosomes and, hence, to grow in macrophages. Values were recorded 1 and 24 h after infection (Fig. 1a). We analyzed, in parallel, the corresponding exometabolomes of these cells to get further insight into metabolite exchanges (consumption/production) between cells and medium (Fig. 1b).

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bacteria; and (iii) the average volume of a bacterial cell is much lower than that of a macrophage, in the range of 1–2×10⁻¹² cm³ per bacterium or 1–4×10⁻⁹ cm³ per macrophage cell. Thus, it is reasonable to assume that the amount of metabolites contained in bacterial cells, in the samples analyzed, do not significantly contribute to the overall amounts measured.

We next performed the quantitative metabolomics analysis of the cell culture supernatants by nuclear magnetic resonance (NMR) to follow the evolution of the exometabolome during growth. This allowed us, by subtraction of concentrations measured at 24 h to those measured at 1 h. Absolute concentrations changes are expressed in mM. Positive/green values correspond to metabolites accumulated in the media, negative/red values correspond to metabolites consumed by cells from the media between 1 and 24 h of incubation. Analyses were performed on biological triplicates and each sample was run in technical triplicates (mean and SD of metabolite concentrations were calculated using R 3.2.3, R Foundation for Statistical Computing, Vienna, Austria. URL: http://www.R-project.org/)

**Fig. 1** Carbon isotopologue distribution of central metabolites in BMMs. a Absolute concentrations of central metabolites in intracellular cell extracts (in µmol L⁻¹) after 24 h of cultivation of BMM macrophages: NI (n = 1); or infected either with wild-type *F. novicida* (WT) (n = 3) or ΔFPI (ΔFPI) strain (n = 3). Fumarate measurements (Fum) represent MS peak area instead of absolute concentrations. b Consumed and produced metabolites, measured in extracellular media in the different infection conditions, profiled by NMR. Mean concentration changes, presented here by subtracting concentration measured at 24 h to those measured at 1 h. Absolute concentrations changes are expressed in mM. Positive/green values correspond to metabolites accumulated in the media, negative/red values correspond to metabolites consumed by cells from the media between 1 and 24 h of incubation. Analyses were performed on biological triplicates and each sample was run in technical triplicates (mean and SD of metabolite concentrations were calculated using R 3.2.3, R Foundation for Statistical Computing, Vienna, Austria. URL: http://www.R-project.org/)

The metabolic modifications recorded in infected macrophages, and especially the glucose consumption, highlighted the importance of metabolic adaptation of *Francisella* for its intracellular survival and multiplication. We focused here on the role of the FBA in *Francisella* pathogenesis. This central enzyme of glycolysis and gluconeogenesis pathways (Supplementary Fig. 1) is at the crossroad of several other metabolic pathways that involve metabolites derived from glycolysis.

**FBA is required for growth on gluconeogenic substrates.** The FBA protein of *F. novicida* (FTN_1329) shows 99.2–99.4% amino acid sequence identity with its orthologues in other *F. tularensis* subspecies (Supplementary Fig. 3). FBA is also highly conserved in multiple other bacterial pathogens. For example, it shares 81.6% identity with *Pseudomonas aerugionosa* (PAO555) FBA, one of its closest homologs, and 74.3% with *Neisseria meningitidis* (NMA0587) FBA. However, it has only modest homology with FBAs of *Mycobacterium tuberculosis* (TBMG_00367) and *E. coli*
K12 (eco: b2925), with 27 and 25.6% amino acid identity, respectively. The FBA protein sequence does not contain any secretion-export signal and is predicted to be cytosolic. The Francisella proteins possess two signature motifs of FBA II according to Prosite database (PS00602 and PS00806) and bear a conserved C-terminal lysine like the FBA of N. meningitidis, suggesting that they are genuine IIB FBA (Supplementary Fig. 3).

We constructed an isogenic deletion mutant of fba in F. novicida by allelic replacement (Methods section) and first evaluated the impact of fba inactivation on bacterial growth in chemically defined medium (CDM) lacking glucose and supplemented with various carbohydrates (Fig. 2). We included in these assays a ΔglpX mutant lacking the strictly gluconeogenic enzyme fructose biphosphatase (FBPase; Supplementary Fig. 1). The Δfba mutant (like the ΔglpX mutant) was unable to grow in media supplemented with gluconeogenic substrates such as pyruvate, glycerol, or succinate, a metabolite of the TCA cycle. In contrast, the Δfba mutant showed only moderate growth defect in the presence of the glycolytic substrates glucose and fructose, as well as in the presence of N-acetylglucosamine (an amino sugar used for the synthesis of cell surface structures and entering the glycolytic pathway after its conversion into fructose-6P). WT growth was restored in the presence of both glucose and glycerol, confirming that FBA is required for growth of F. novicida in culture, mainly when gluconeogenic substrates are used as carbon sources.

It is likely that glycolytic substrates can use alternate route in the Δfba mutant, and in particular the pentose-phosphate pathway (PPP). Indeed, we have recently demonstrated, using 13C-labeled glucose, the recycling of carbons through the PPP in WT F. novicida. In contrast, when 13C-labeled pyruvate was used, compounds of the PPP were not detected.

Of note, whereas the Δfba mutant was unable to grow on ribose (a product of the PPP), the ΔglpX mutant showed WT growth on this sugar. WT growth was always restored in the Δfba-complemented strain, confirming quantitative reverse transcription PCR (qRT-PCR) analyses, which demonstrated the absence of polar effect of the mutation (Supplementary Fig. 4). Since ribose cannot be converted into glucose-6P because the oxidative branch of the PPP is irreversible, FBA seems to be the only way for ribose to feed gluconeogenesis.

**fba inactivation impairs virulence.** We next evaluated the impact of fba inactivation in vitro, on intracellular multiplication; and in vivo, on virulence in the mouse model. The ability of wild-type F. novicida (WT) and Δfba strains to survive and multiply in murine macrophage-like J774.1 cells was determined in cell culture medium supplemented either with glucose or glycerol or supplemented with both glucose and glycerol (Fig. 3a–c). We used a ΔFPI mutant strain as a negative control. In standard Dulbecco’s modified eagle’s medium (DMEM; i.e., supplemented with glucose), the intracellular multiplication of the Δfba mutant was moderately affected (8–10-fold reduction of bacterial counts at 10 and 24 h, as compared to WT; Fig. 3a). In contrast, when glucose was substituted by glycerol, multiplication of the Δfba mutant was severely impaired (Fig. 3b). The Δfba mutant already showed a 10-fold reduction of intracellular bacteria compared to cells infected with the WT strain at 10 h; and a 1000-fold reduction of bacterial counts were recorded at 24 h (comparable to the ΔFPI mutant). Remarkably, when the medium was simultaneously supplemented with glucose and glycerol, the Δfba mutant multiplied like the WT strain (Fig. 3c). Functional complementation (i.e., introduction of a plasmid-born WT fba allele into the Δfba mutant strain) always restored WT growth.
The intracellular behavior of the \( \Delta fba \) mutant was also tested in BMMs from BALB/c mice. Comforting the observations in J774-1 cells, growth of the \( \Delta fba \) mutant was identical to that of the WT at 10 h and only slightly impaired (fivefold less bacterial counts) at 24 h (Fig. 3d) when BMMs were supplemented with glucose. In contrast, intracellular multiplication of the \( \Delta fba \) mutant was severely impaired (Fig. 3e) when the culture medium was supplemented either with glucose or glycerol, or an equimolar concentration of glucose and glycerol. Functional complementation restored normal intracellular bacterial replication in both cell types, in the presence of glucose. In the presence of glycerol, complementation was fully restored up to 10 h and only partially at 24 h. Altogether these assays demonstrate that the \( \Delta fba \) mutant is unable to multiply intracellularly when gluconeogenic substrates are used as carbon sources (Figs. 2–3). In contrast, when glycolytic substrates are used, the growth defect of the \( \Delta fba \) mutant is essentially suppressed.

We confirmed that the presence of the empty vector (pKK214) in WT and \( \Delta fba \) mutant strains used in these experiments had no impact on the phenotypes observed (Supplementary Fig. 5).

Interestingly, it has been recently shown that macrophages stimulated with the PPAR\( \beta/d \) agonist GW0742 increased their intracellular concentration of glucose\(^{32} \). This prompted us to test whether the addition of GW0742 to cell culture medium would result in increased intracellular bacterial multiplication of the \( \Delta fba \) mutant. Addition of GW0742 had no visible effect on the multiplication of the \( \Delta fba \) mutant in glycerol-grown J774-1 cells, during the first 10 h. However, a 15-fold increase in the number of intracellular \( \Delta fba \) mutant bacteria was recorded at 24 h.
Bacteria were plated on chocolate agar plates at different time points and viable bacteria were numerated 2 days after. Experiments were realized three times and Δ14 and 22% of co-localization was recorded with WT of FBA in cells (Fig. 3g, Supplementary Fig. 7A), after 1 h, most of Δfba mutant bacteria no were no longer associated with LAMP-1 (~13 phages were infected with either WT Δfba strain (the isogenic ΔFPI mutant was used as a negative control). Bacteria and the late phagosomal marker LAMP-1 were labeled with specific antibodies and their co-localization was monitored at three time points (1, 4, and 10 h). In glycerol-supplemented cells (Fig. 3g, Supplementary Fig. 7A), after 1 h, most of Δfba mutant bacteria no were no longer associated with LAMP-1 (~13 and 21% of co-localization recorded with WT F. novicida and Δfba mutant, respectively) and co-localization of the Δfba mutant remained low after 4 h (~14%) and after 10 h (~15%). In glucose-grown cells (Supplementary Fig. 7B), after 1 h of infection, only 14 and 22% of co-localization was recorded with WT F. novicida and Δfba mutant, respectively, indicating that the Δfba mutant was able to escape phagosomes as fast as WT F. novicida. Co-localization of the Δfba mutant remained still very low after 4 h (~10%) and after 10 h (~12%). In contrast, the ΔFPI mutant strain remained trapped into phagosomes, as illustrated by high co-localization with LAMP-1 at all time points tested (80, 85, and 88%, after 1, 4, and 10 h, respectively). Altogether, these results indicate that the intracellular growth defect of the Δfba mutant, observed in glycerol-grown conditions, was not due to altered phagosomal escape but to impaired cytosolic multiplication.

Finally, to estimate the impact of fba inactivation on bacterial virulence, we performed an in vivo competition assay between WT and Δfba mutant bacteria, in 7-week-old female BALB/c mice and monitored the bacterial burden in spleen and livers 2.5 days after infection by the ip route (Fig. 3h). The competition index recorded for the Δfba mutant was very low in both target organs (between 10^-3 and 10^-4), demonstrating the importance of FBA in Francisella virulence in the mouse model.

**fba inactivation increases resistance to oxidative stress.** Upon entry into cells, Francisella transiently resides in a phagosomal compartment that acquires bactericidal reactive oxygen species (ROS). We therefore examined the ability of WT F. novicida and Δfba mutant strains to survive under oxidative stress conditions. We included in these assays a ΔglpX mutant as a control to evaluate the contribution of gluconeogenesis to oxidative stress. Bacteria were first grown in CDM supplemented with glucose + glycerol and then exposed to either to H2O2 (Fig. 4a, b), the organic hydroperoxides tert-butyl hydroperoxide (TBH, 10 mM) or cumene hydroperoxide (CH, 10 mM) or cumene hydroperoxide (CH, 10 mM) (Fig. 4c, d) for 1 h. In the two H2O2 conditions tested, the viability of both WT, ΔglpX and Δfba-complemented strains was equally affected and behaved like the WT strain (Fig. 4a, b). In contrast, the Δfba mutant was systematically more resistant to these stresses than the other strains. Indeed, after 30 min of exposure to H2O2, the viability of the Δfba mutant was essentially unaffected whereas that of the other strains showed a 4 logs decrease in the number of viable bacteria. The Δfba mutant showed also a greater resistance to organic hydroperoxides (CH and TBH), especially to TBH compared to WT. Of note, the FBA overproducing strain (WT strain bearing a plasmid carrying the WT fba presented) was equally affected as the WT strain, demonstrating the importance of FBA in oxidative stress resistance beyond its role in gluconeogenesis. We next examined the resistance of the mutant to other stresses: acidic, SDS, or upon incubation in the presence of 10% human serum. Under all the conditions tested, the viability of Δfba mutant strain was undistinguishable from that of the parental strain (Supplementary Fig. 8).

The bactericidal activity of macrophages, and notably ROS production, is increased in the presence of IFNγ. We therefore tested the intracellular multiplication of the Δfba mutant compared to WT bacteria, in IFNγ-stimulated J774.1 macrophages. The Δfba mutant showed improved survival and/or intracellular multiplication in the presence of IFNγ compared to WT (up to 20-fold increase in bacterial counts after 24 h), supporting the notion that fba inactivation conveys to Francisella...
an increased resistance to the ROS stress induced upon IFNγ treatment (Supplementary Fig. 9).

A role for FBA in transcription. The apparent specific impact of fba inactivation on oxidative stress resistance prompted us to first test the expression of the unique gene, katG, encoding the catalase responsible for the detoxification of H₂O₂ into H₂O and O₂. The transcription of katG was quantified in WT and Δfba mutant strains, in broth as well as in infected macrophages (Fig. 5). qRT-PCR analyses revealed that fba inactivation provoked a 12-fold increase of katG gene transcription in tryptic soya broth (TSB) (Fig. 5a) and complete inactivation of fba in the Δfba mutant was confirmed (Fig. 5b). Catalase activity was also measured in whole-cell lysates from bacteria grown in the same TSB medium (Fig. 5c). Supporting the qRT-PCR data, increased catalase activity was measured in whole-cell lysates from bacteria grown in the same TSB medium (Fig. 5c). Supporting the qRT-PCR data, increased catalase activity was measured in whole-cell lysates from bacteria grown in the same TSB medium (Fig. 5c). Supporting the qRT-PCR data, increased catalase activity was measured in whole-cell lysates from bacteria grown in the same TSB medium (Fig. 5c). Supporting the qRT-PCR data, increased catalase activity was measured in whole-cell lysates from bacteria grown in the same TSB medium (Fig. 5c).

It has been shown that a ΔkatG mutant of Francisella provoked the rapid cytosolic accumulation of H₂O₂, triggering an inflammatory reaction via Ca²⁺ signaling. This katG-dependent control on the available intracellular H₂O₂ pool was proposed to control the production of pro-inflammatory cytokine and increased IL-6 production. We therefore thought to monitor the amount of IL-6 secreted in the supernatant of J774-1 macrophages, infected either with WT or with the Δfba mutant (Fig. 5e). Supernatant from NI cells was used as negative control. As expected, IL-6 secretion, measured by ELISA, was significantly higher in the supernatant of WT-infected cells compared to NI cells. Remarkably, IL-6 secretion was also significantly higher in the supernatant of WT-infected cells than in the supernatant of Δfba-infected cells.

Intracellular bacterial counts, performed on the same cells as controls (Fig. 5f), confirmed that comparable numbers of intracellular bacteria were present in WT and Δfba mutant at 24 h. These data are in agreement with a direct correlation between FBA-mediated katG repression and increased inflammatory response.

We next compared the amount of ROS in WT-infected cells to that in Δfba-infected cells, 10 and 24 h after infection. For this, we used the 2′,7′-dichlorofluorescin diacetate (DCFDA) Cellular ROS Detection Assay Kit (Abcam, Cambridge, UK). The ROS content was ~20% lower with the Δfba mutant compared to WT, at both time points (Supplementary Fig. 10A). As positive control, NI cells were stimulated with 5 µg mL⁻¹ of lipopolysaccharide from E. coli K12 (LPS-EK) Standard. LPS stimulation provoked an increase of ROS production that was up to 1.5-fold higher than that in WT-infected cells, at both time points tested. DCFDA levels were also visualized using fluorescence microscopy. The percentage of fluorescent cells was significantly higher in WT-infected cells (33%) or LPS-stimulated NI cells (63.5%) than in Δfba-infected cells (8.5%) (Supplementary Fig. 10B).

Altogether, these assays further supported a direct correlation between the FBA-mediated repression of KatG expression and cellular ROS production. These data led us to hypothesize that FBA functions might extend beyond metabolic functions. To check whether fba inactivation could have a broader impact on protein expression than solely affecting KatG expression, we next performed a whole-cell comparative nanoLC-MS/MS proteomic analysis of WT, Δfba, and FBA-overproducing (WT-cp), strains.

Fig. 5 Quantitative real-time RT-PCR analysis. a, b Bacteria were grown overnight in TSB and qRT-PCR analyses were performed on selected genes, a katG gene or b fba gene in wild-type F. novicida, Δfba mutant, and fba-complemented (Cp-fba) strains. For each gene, the transcripts were normalized to helicase rates (FTN_1594). c Catalase activity assay was repeated at least three times. Mean and SD of three wells of one typical experiment are shown. d, e Intracellular bacterial multiplication of wild-type F. novicida (WT) and Δfba mutant. Total RNA was analyzed by quantitative RT-PCR with katG and fba gene. For each gene, the transcripts were normalized to helicase rates (FTN_1594). f Intracellular bacterial multiplication of wild-type F. novicida (WT) and Δfba mutant was determined at 24 h, in the infected J774-1 cells used for the IL-6 dosage, as a control. **p < 0.01. a-c mean and SD of three independent experiments are shown; d-f each assay was repeated at least three times. Mean and SD of three wells of one typical experiment are shown. p-values were determined by the two-tailed unpaired Student’s t-test.
We could identify 1372 proteins across the 9 samples analyzed, which represent 80% of the predicted \( F.\) tularensis proteome. For statistical analysis, we kept 1154 proteins robustly quantified (data are available via ProteomeXchange with identifier PXD006908). An ANOVA test identified 26 proteins whose amount significantly differed between the three strains. Of note, most of them were conserved in the subspecies \( holarctica\) and \( tularensis\). These 26 proteins were submitted to biclustering analysis and represented in the heatmap (Fig. 6). The column tree revealed that the protein profiles of the WT and the WT-cp strains were different from the \( \Delta fba\) mutant.

Two opposite patterns could be distinguished: (i) a group of 19 proteins were expressed in lower amount only in the \( \Delta fba\) mutant strain, suggesting that they are (directly or indirectly) positively regulated by FBA; and (ii) two proteins were in higher amount only in the \( \Delta fba\) mutant strain, suggesting that they are (directly or indirectly) negatively regulated by FBA. The largest group of proteins under-represented or absent in the proteome of the \( \Delta fba\) mutant comprised (in addition to FBA itself) proteins belonging to various functional categories, such as ribosomal proteins, a putative DNA binding protein and RpoA2, one of the \( \alpha\) subunits of RNA polymerase. Remarkably, fully supporting the transcriptional data, the catalase KatG was one of the two proteins expressed in higher amounts only in the \( \Delta fba\) mutant strain (together with a putative Heme binding protein). Of note, the only down-regulated proteins in the FBA overproducing strain comprised two proteins of the same operon (FadA and FadB) involved in fatty acid degradation, and two proteins linked to lysine metabolism. The fact that these pathways might also be affected by the over-production of FBA, suggest that FBA functions might extend beyond the proteins analyzed in this study.

In order to directly probe a role of FBA in transcription regulation, we constructed a His-tagged version of FBA, by fusing the His tag to its C-terminus (FBA-HA). The FBA-HA protein expressed in \( \Delta fba\) was used to detect the in vivo binding of FBA to the promoters of genes corresponding to proteins whose expression varied over twofold in the proteomic comparison between WT and \( \Delta fba\) mutant strains (Fig. 7). We performed chromatin immunoprecipitation (ChIP) followed by qRT-PCR analysis on two sets of genes: (i) two genes corresponding to proteins expressed in higher amounts in the \( \Delta fba\) mutant (kat\( G\) and hemeBP); and (ii) three genes corresponding to proteins expressed in lower amounts in the \( \Delta fba\) mutant (rpoA, uvrB, and fadA) (Fig. 7a). We observed a nearly 30-fold enrichment of the kat\( G\) promoter region and a fivefold enrichment of hemeBP promoter region. A nearly 10-fold enrichment of the rpoA promoter region was observed but no–or only very modest–enrichment was observed with the two other promoter regions (i.e., uvrB and fadA) (Fig. 7a). Transcription of rpoA was also significantly higher in the WT (>80-fold) and \( fba\)-complemented strains compared to the \( \Delta fba\) mutant, suggesting
a direct role of FBA on rpoA transcription activation (Supplementary Fig. 11).

Transcription of seven additional genes, corresponding to proteins positively controlled by FBA, was also tested by qRT-PCR. Corroborating the proteomic analyses, transcription of these genes was higher in the WT and fba-complemented strains than in the Δfba mutant. In particular, transcription of four of them (pyrG, 3OS 5S, glyS, and pheT) was 50-fold to 200-fold higher in the WT strain than in the Δfba mutant (Supplementary Fig. 11). We also monitored qRT-PCR, katG and rpoA gene expression in a ΔglpX mutant compared to WT F. novicida. Comparable expression of these two genes was recorded in the two strains, confirming that GlpX was not involved in their transcriptional control (Supplementary Fig. 11).

Electrophoretic mobility shift assays (EMSAs) were then performed to confirm direct binding of FBA to katG and rpoA promoter regions (Fig. 7b). For this, DIG-labeled double-stranded DNA fragments, corresponding to the regions immediately preceding each coding sequence (designated pKatG and pprA, respectively) were incubated in the presence of purified His-tagged FBA (FBA-HA) recombinant protein. With pKatG, a single band was detected with the labeled probe alone. Upon incubation with FBA-HA, a fraction of the probe was shifted and this shift was almost completely suppressed when a 125-fold excess of competing unlabeled specific pKatG oligonucleotide was added to the reaction (in addition to the labeled specific probe). With pprA, two bands were detected with the labeled probe alone, possibly corresponding to different conformations of the probe. A major shifted additional band was detected upon incubation with FBA-HA as well as faint upper bands. The shifted bands disappeared when a 125-fold excess of competing unlabeled specific oligonucleotide was added to the reaction (Fig. 7b).

Finally, transcriptional pkatG-lacZ and prpoA-lacZ fusion were constructed and expressed in WT F. novicida or in Δfba isogenic mutant strain (Fig. 7c). As expected, the β-galactosidase activity recorded with the pkatG-lacZ construct was 3.5-fold higher in the Δfba mutant background compared to WT. Conversely, the β-galactosidase activity recorded with the prpoA-lacZ construct was 2.2-fold lower in the Δfba mutant background compared to WT.

Altogether, these assays confirmed the specificity of the interaction between FBA and katG, and rpoA promoter regions and supported that FBA specifically binds to different promoter regions, contributing either to transcriptional activation or repression.

Discussion
Recent studies have shown that amino acids are likely to represent major carbon sources for intracellular Francisella and serve as gluconeogenic substrates.31–38, 41–43 We show here that FBA is not involved in phagosomal escape but is critical for cytosolic multiplication in the presence of gluconeogenic substrates. Our metabolomics analysis performed on BMMs revealed that the infection by Francisella triggered numerous variations of intracellular metabolites, translating a complex metabolic response of the host to infection. Remarkably, a strong reduction of glucose present in the cell culture supernatant, with a concomitant increase of intracellular glucose-6P, was recorded after 24 h in infected BMMs, reflecting an increase of glucose consumption by

![Fig. 7 ChIP-qPCR, EMSA, and β-galactosidase assays. a ChIP-qPCR experiments were performed with Δfba strain expressing a His-tagged version of FBA (Δfba/cpFBA-HA) and with wild-type F. novicida (WT, negative control). The results are expressed as relative enrichment of the detected fragments. Mean and SD of three independent experiments are shown. Five promoter regions were tested: two (blue columns) corresponding to down-regulated proteins (according to the proteomic analysis) i.e., katG (KatG) and hemeBP (FTN_0032 or Heme Binding Protein); and three (red columns) to up-regulated proteins i.e., rpoA (RNA polymerase α subunit), uvrB (Endonuclease ABC subunit B), and fadA (Fatty acid degragation). b Electrophoretic mobility shift assays (EMSA) analysis of FBA—pKatG (left) and FBA—pproA (right) promoter interactions. EMSA assays were performed with DIG-labeled katG and rpoA promoter regions (pKatG, 200 bp; prpoA, 220 bp) and purified His-tagged FBA (FBA-HA) recombinant protein. Lane 1: labeled probe alone; lane 2, labeled probe incubated with 0.8 µg purified FBA-HA; lanes 3: probe incubated with 0.8 µg purified FBA-HA in the presence of 125-fold excess of corresponding unlabeled probe. The gray arrows (to the left of each panel) indicate the migration of the probe alone and the black arrows (to the right), the shifted bands. As negative control (right panel), EMSA assays were performed with DIG-labeled uvrD promoter region (uvrD, 188 bp). Lane 1: labeled probe alone; lane 2, labeled probe incubated with 0.8 µg purified FBA-HA; lanes 3: probe incubated with 0.8 µg purified FBA-HA in the presence of 125-fold excess of corresponding unlabeled probe. As expected, no specific shifted bands were observed with this promoter region in presence of purified FBA-HA. c Quantification of lacZ expression in F. novicida wild type (WT) and Δfba mutant strains containing either pKatG (left) or prpoA (right) promoter regions by β-galactosidase assay, as measured in Miller units. Each assay was repeated at least three times. Mean and SD of three wells of one typical experiment are shown. (p-values were determined by the two-tailed unpaired Student’s t-test, ***p < 0.0001)
these cells. In contrast to *L. monocytogenes*, which possesses a specific hexose phosphate transporter (Hpt) required for virulence, *Francisella* cannot use the cytosolic pool of glucose-6P directly as a glycolytic substrate since its lacks a dedicated transporter. In this glucose-restricted context, a fully functional gluconeogenic pathway is thus likely to be critical for the bacteria to allow the utilization of alternative available nutrients (such as amino acids, glycerol, or other carbohydrates). The intracellular concentrations of all the measured TCA cycle metabolites decreased in infected BMMs. This could be due to their increased consumption by the cell in response to the infection and contributed by the multiplying bacteria themselves. In spite of that, the level of intracellular ATP appeared to have increased by at least twofold in WT-infected BMMs whereas it remained unchanged in Δ*FPI*-infected BMMs compared to NI macrophages. Other sources of ATP generation have been very recently described which would account for the maintenance of a sufficient ATP pool in these cells. For comparison, infection with *L. monocyto genes* has been shown to increase glycolytic activity, enhance flux of pyruvate into the TCA cycle in infected BMMs and favor glucose uptake by infected cell and the production of compounds, such as glucose-6P, serine, and glycerol.

Altogether, these observations are compatible with the notion that cytosolic multiplying bacteria may take advantage of the glycolytic activity of the host to obtain a valuable source of metabolites for their own intracellular nutrition. The requirement of a functional FBA enzyme, for growth in the presence of gluconeogenic substrates and for virulence in the mouse, suggests that in vivo *Francisella* does not encounter the proper combination of carbon sources that could compensate for the lack of FBA.

*Francisella* produces several antioxidant enzymes such as the superoxide dismutases SodB and SodC, and the catalase KatG. In addition to these primary antioxidant enzymes, other proteins have also been shown to contribute (directly or indirectly) to oxidative stress defense, such as the alkyl-hydro-peroxide reductase AphC, proteins with sequence similarities to the Organic hydperoxide resistance protein (Ohr) as well as the MoxR-like ATPase. The pleiotropic regulator OxyR is a primary regulator of oxidative stress in many bacteria that responds to peroxides (H₂O₂). In *Francisella*, inactivation of oxyR confers high sensitivity to oxidants, deficient intramacrophage growth, and attenuated virulence in mice. OxyR was also shown to bind to the upstream promoter regions of *katG*, indicating that *katG* expression is under the control of multiple regulatory mechanisms.

An earlier transcriptomic study, carried out in BMMs infected with *F. tularensis* Schu S4 strain, has shown that *katG* transcription was expressed during the first two hours after bacterial entry and was later shut down for the rest of the infectious cycle whereas that of *fba* was induced after 4 h of infection and remained high up to 24 h. In agreement with this report, and further supporting the notion that FBA negatively regulates the expression of *katG* in infected cells, our qRT-PCR analysis (Fig. 5d) showed that *katG* expression was 7-fold up-regulated in the Δ*fba* mutant compared to WT, at 24 h of infection, in J774-1 macrophages supplemented with glucose and glycerol. These data suggest that the up-regulation of *katG* in the Δ*fba* mutant is not critical for intracellular multiplication in these conditions (in which the Δ*fba* metabolic defect is fully bypassed by appropriate carbohydrate supplementation). A role at later stages of the infectious cycle (such as cell to cell dissemination) and in other cell types is however possible.

ROS generated by macrophages upon infection have been shown to act as microbicidal effector molecules as well as secondary signaling messengers that regulate the expression of various inflammatory mediators. Of interest, the antioxidant action of the *katG*-encoded catalase of *Francisella* contributes to maintain the redox homeostasis in infected macrophages. The rapid cytosolic accumulation of H₂O₂, recorded in macrophages infected with a ΔkatG mutant (i.e., lacking catalase activity), triggers a strong inflammatory reaction mediated by Ca²⁺ entry via the transient receptor potential melastatin 2 (TRPM2) channels. Indeed, TRPM2-dependent Ca²⁺ accumulation is limited and IL-6 production remains low.

The control of *katG* expression, mediated by FBA in the host cytosol, might constitute a mean of fine-tuning the redox status of the infected cell, leading to the control of pro-inflammatory cytokine and IL-6 production. Supporting this notion, we found that the total amount of ROS produced in cells infected with the Δ*fba* mutant (in which *katG* expression is not repressed) was lower than in cells infected with the WT strain (Supplementary Fig. 9). Interestingly, previous studies have shown that *Francisella* also secreted KatG into the macrophage cytosol upon infection as well as in the supernatant of bacterial cultures. Thus, KatG-dependent H₂O₂ detoxification might occur not only upon bacterial adsorption but might also be due to the direct action of the enzyme released in the host cytosol.

Altogether, these data are compatible with a sequential model of regulation (Fig. 8): (i) in the phagosomal compartment, *katG* expression is up-regulated due to the accumulation of H₂O₂ (designated pH₂O₂, derived from ROS produced by the NADPH oxidase) and FBA is not or poorly expressed; (ii) upon cytosolic escape, WT *Francisella* initially scavenges cytosolic H₂O₂, limiting...
the activation of the calcium channel TRMP2 and delaying the Ca\(^{2+}\) dependent inflammatory response\(^{34}\); (iii) the progressive increase of \(fba\) expression in actively multiplying bacteria leads to the concomitant reduction of \(katG\) expression; (iv) the accumulation of cytosolic \(H_2O_2\) then stimulates the TRMP2-dependent entry of \(Ca^{2+}\); and (v) ultimately leads to an inhibited. In this context, the cytosolic concentration of \(H_2O_2\) remains low and ultimately triggers only reduced IL-6 production in infected cells compared to WT bacteria.

Hence, FBA-mediated control of \(KatG\) expression should not be seen as a specific pathophysiologic role to render \(Francisella\) more vulnerable to a pro-inflammatory response but rather as a means for the bacterium to combine metabolism and transcriptional regulation to optimally modulate the redox status of the infected cell. Indeed, one should bear in mind that intracellular survival and dissemination of the pathogen relies on its capacity to counteract both nutritional and innate immunity through an adaptation to the available nutritional resources and a tight and temporally-controlled dampening of cytokine production\(^{60}\), ultimately leading to inflammammasome activation and pyroptosis, allowing bacterial release and dissemination to adjacent cells.

Proteins that perform two or more distinct biological functions have been designated moonlighting proteins\(^{61}\) and FBA can certainly be considered as a member of this family\(^{21}\). However, the only moonlight activity of FBA described thus far in pathogenic bacteria was a role as a secondary adhesin. For example, FBA is an essential enzyme in \(M. tuberculosis\) and also binds human plasminogen. Pneumococcal FBA is involved in the binding to human lung epithelial A549 cells via an interaction with a receptor belonging to the cadherin family. In \(N. meningitidis\), FBA is not essential but is required for optimal adhesion to both human epithelial and endothelial cells\(^{62, 63}\). The recent detection of FBA in the cell wall fraction of \(Coxiella burnetii\)\(^{64}\) and the identification of FBA orthologues in other pathogenic Gram-negative bacterial species, suggest that translocation of FBA to the Gram-negative cell envelope might constitute a more generalized phenomenon.

A putative role of metabolic enzymes in transcription regulation has already been suggested in bacteria, yeast as well as in eukaryotic cells. For example, in pathogenic \(Streptococcus pyogenes\), the tagatose-1,6-\(\beta\)-biphosphoaldolase LacD.1 acts as a negative regulator of the gene encoding the secreted cysteine protease SpeB\(^{65}\). This led to suggest that it might be a mechanism used by the bacterium to couple essential transcription programs to the sensing of its nutritional environment. In the yeast \(Saccharomyces cerevisiae\), the class II FBA, in addition to its function in glycolysis, interacts physically with RNA polymerase III and plays a role in the control of its transcription\(^{66}\). In mammalian tissues, several enzymes involved in the glycolytic pathway display various non-glycolytic functions such as DNA repair and transcription, and are thus involved in important cellular functions including apoptosis, cell cycle control, and signaling pathways\(^{67}\).

Our proteomic analyses showed that FBA exerted a specific repressive effect on only two proteins, including the catalase \(KatG\). FBA appeared to be also involved in the up-regulation of multiple proteins including the \(\alpha_2\) subunit of RNA polymerase (RNAP). Since the \(\alpha\) subunit is a common site of interaction of RNAP with transcription activator proteins\(^{68}\), affecting its expression is likely to alter transcription. Intriguingly, the \(Francisella\) genomes have the unique property to encode two different \(\alpha\) subunits of RNAP with different regions predicted to be critical for dimer formation, promoter recognition, and activator interaction, suggesting possible distinct roles for the two \(\alpha\) subunits in transcription regulation. Beyond this, our data also suggest that FBA might be involved in other pathways such as fatty acid metabolism. The role of metabolic enzymes in the regulation of non-metabolic functions in pathogenic microorganism should deserve renewed attention.

### Methods

#### Ethics statement

All experimental procedures involving animals were conducted in accordance with guidelines established by the French and European regulations for the care and use of laboratory animals (Decree 86–848, 2001–464, 2001–486 and 2001–131, and European Directive 2010/63/UE) and approved by the INSERM Ethics Committee (Authorization Number: 75-906).

#### Strains and culture conditions

All strains used in this study are derivative from \(F. tularensis\) subsp. \(novicida\) U112 (\(F. novicida\) WT) and are described in Supplementary Table I. Strains were grown at \(37^\circ\)C on pre-made chocolate agar PolyViteX plates (BioMerieux), TSB or CDM supplemented with the appropriate carbon source at a final concentration of 25 mM. The CDM used for \(F. tularensis\) subsp. \(novicida\) corresponds to standard CDM\(^{68}\) without threonine and valine\(^{69}\).

For growth condition determination, bacterial strains were inoculated in the appropriate medium at an initial OD\(_{600}\) of 0.05 from an overnight culture in TSB.

#### Stress assays

Stationary phase bacterial cultures were diluted to a final OD\(_{600}\) of 0.1 in TSB broth. Exponential-phase bacterial cultures were diluted to a final concentration of 10\(^{10}\) bacteria per mL and subjected to either 500 \(\mu\)M \(H_2O_2\), 10 mM \(H_2O_2\), 10 mM Tertbutyl hydroperoxide, 10 mM Cumene hydroperoxide (1 h); pH 5.5 (1 h); 0.05% SDS (4 h); or 10% human serum (1 h). The number of viable bacteria was determined by plating appropriate dilutions of bacterial cultures on PolyViteX plates at the start of the experiment and after the indicated durations. Cultures (5 mL) were incubated at \(37^\circ\)C with rotation (100 rpm) and aliquots were removed at indicated times, serially diluted and plated immediately. Bacteria were enumerated after 48 h incubation at \(37^\circ\)C. Experiments were repeated independently at least twice and data represent the average of all experiments.

#### Construction of chromosomal deletion mutants

We inactivated the gene \(fba\) in \(F. novicida\) (FTn_1239) by allele replacement resulting in the deletion of the entire gene (start and three last codons were conserved). We constructed a recombinant PCR product containing the upstream region of the gene \(fba\) (FBA-UP), a kanamycin resistance cassette (\(nptII\) gene fused with \(pGro\) promoter) and the downstream region of the gene \(fba\) (FBA-DN) by overlap PCR. Primers (Supplementary Table I) FBA upstream forward (p1) and FBA upstream (spl, K7) RV (p2) amplified the 505 bp region upstream of position +1 of the FBA-encoding sequence (FBA-UP), primers \(pGro\) FW (p3) and \(nptII\) RV (p4) amplified the 1091 bp kanamycin resistance cassette (\(nptII\) gene fused with \(pGro\) promoter); and primers FBA downstream (spl, K7) FW (p5) and FBA downstream RV (p6) amplified the 559 bp region downstream of the position +1087 of the \(FBA\) gene (FBA-DN). Primers p2 and p5 have an overlapping sequence of 12 and 12 nucleotides with primers p3 and p4, respectively, resulting in fusion of FBA-UP and FBA-DN with the cassette after crossing-over PCR. All single-fragment PCR reactions were realized using Phusion High-Fidelity DNA Polymerase (ThermoScientific) and PCR products were purified using Nucleospin\(^{\text{®}}\) Gel and PCR Clean-up kit (Macherey-Nagel). Overlap PCRs were realized using 100 ng of each purified PCR products and the resulting fragment of interest was purified from agarose gel. This fragment was then directly used to transform wild type \(F. novicida\) by electroporation\(^{68}\). Recombinant bacteria were isolated by spreading onto Chocolate agar plates containing kanamycin (5 \(\mu\)g mL\(^{-1}\)). The mutant strains were checked for loss of the wild type gene \(FBA\) by PCR product direct sequencing (GATC-biotech) using appropriate primers.

#### Functional complementation

The plasmid used for complementation of the \(F. novicida\) \(\Delta fba\) mutant (\(\Delta fba\)), pKK-FBA\(_{\text{pGro}}\), is described below. Primers \(pGro\) FW and \(pGro\) RV amplified the 328 bp of the \(pGro\) promoter and primers BAFW/FBA (\(PstI\)) RV amplified the 1064 bp FBA gene from U112. PCR products were purified and \(PstI\) (\(PstI\) promoter) or \(PstI\) (\(PstI\) FBA) restricted in presence of FastAP Thermosensitive Alkaline Phosphatase (ThermoScientific) to avoid self-ligation. A mixture of \(pGro\) promoter and interest gene fragments was then incubated in T4 Polynucleotide Kinase to allow blunt end ligation and fragments were then cloned in pKK214 vector after Smal/PstI double digestion and transformed in E. coli TOP10. Recombinant plasmid pKK-FBA\(_{\text{pGro}}\) (designated \(Cp-fba\)) was purified and directly used for chemical transformation in \(F. novicida\) U112\(^{12}\) by electroporation. Recombinant colonies were selected on Chocolate agar plates containing tetracycline (5 \(\mu\)g mL\(^{-1}\)) and kanamycin (5 \(\mu\)g mL\(^{-1}\)).

As controls, \(WT\) \(F. novicida\) and \(\Delta fba\) mutant strains, carrying the empty vector pKK214, designated WT(–) and \(\Delta fba\)(–), respectively, were also constructed and tested.

#### Catalase assay

Catalase enzyme activity was analyzed with a Catalase Assay Kit (Abcam, Cambridge, UK). \(WT\) \(F. novicida\) and \(\Delta fba\) strains were grown overnight...
in TSB supplemented with glucose and cysteine. 2 × 10⁸ bacteria were harvested and used for the enzyme assay according to the manufacturer’s instructions. After 30 min, the reaction was followed by the optical density was measured at OD₅₇₀nm with a microplate reader. The assay was repeated twice, with similar results.

**Transcriptional analyses.** Isolation of total RNA and reverse transcription: For transcriptional analyses of bacteria grown in TSB, cultures were centrifuged for 2 min in a microcentrifuge at room temperature and the pellet was quickly resuspended in Trizol solution (Invitrogen, Carlsbad, CA, USA). For transcriptional analyses of bacteria in infected cells, J774-1 cells grown in standard DMEM were infected with either WT F. novicida (WT) or Δfba mutant strain for 24 h. NI cells, incubated in the same conditions, were used as a negative control. Cells were then collected by scraping, centrifuged at max speed in a microcentrifuge at room temperature and the pellet was quickly resuspended in Trizol solution.

Electrophoretic mobility shift assay (EMSA) was carried out using a DIG gel shift kit (Roche Diagnostic Corporation, Indianapolis, IN, USA) with an on-column RNeasy Clean-up protocol (Qiagen, Valencia, CA, USA) followed by the RNAse A treatment. RNA samples were treated with chloroform and the aqueous phase was used in the RNeasy Clean-up protocol (Qiagen, Valencia, CA, USA) with an on-column RNeasy Clean-up protocol (Qiagen, Valencia, CA, USA) with an on-column RNeasy Clean-up protocol (Qiagen, Valencia, CA, USA) with an on-column RNeasy Clean-up protocol (Qiagen, Valencia, CA, USA) with an on-column RNeasy Clean-up protocol (Qiagen, Valencia, CA, USA) with an on-column RNeasy Clean-up protocol (Qiagen, Valencia, CA, USA) with an on-column RNeasy Clean-up protocol (Qiagen, Valencia, CA, USA). Transcriptional analyses of bacteria in infected cells, J774-1 cells grown in standard DMEM were infected with either WT F. novicida (WT) or Δfba mutant strain for 24 h. NI cells, incubated in the same conditions, were used as a negative control. Cells were then collected by scraping, centrifuged at max speed in a microcentrifuge at room temperature and the pellet was quickly resuspended in Trizol solution.

Chromatin immunoprecipitation-qPCR assay (ChIP-qPCR) was performed with wild-type F. novicida (WT) and Δfba strain expressing a His-tagged version of FBA (FBA-HA) (Fba/cpFBA-HA) bearing the 6x-His epitope fused at the C-terminal end of the protein. Bacteria were grown at 37°C in 100 mL TSB supplemented to mid-log (OD₆₀₀nm 0.3–0.4). Then bacteria were incubated in 24-well plates, in 1 mL of fresh medium for 1 h. In parallel, the bacterial suspension was divided by 100-fold excess of formaldehyde (Sigma) was added to a final concentration of 1% formaldehyde (Sigma) for 30 min, after which glycine (Sigma) was added to a final concentration of 125 fold excess of unlabeled probe. The labeled probe was incubated for 30 min:

- 5'-TAAAGGAGGAGAAGCATT-3'
- 5'-TTAAAAACGACGCCTAGT-3'

Amplification reactions were performed using Phusion High-Fidelity DNA Polymerase (ThermoScientific). The amplified fragments were purified using the primer pairs used in the EMSA and input samples) were divided by the following reactions:

- 5'-TAAAGGAGGAGAAGCATT-3'
- 5'-TTAAAAACGACGCCTAGT-3'

Amplification reactions were performed using Phusion High-Fidelity DNA Polymerase (ThermoScientific). The amplified fragments were purified using the primer pairs used in the EMSA and input samples) were divided by the following reactions:

Quantitative real-time RT-PCR: WT F. novicida and mutant strains were grown overnight at 37°C. Then, samples were harvested and RNA was isolated and reverse transcribed. The 25 μl reaction consisted of 5 μl cDNA template, 12.5 μl Fast SYBR Green Master (Roche Diagnostics), and 2 μl 10 mM of each primer and 3.5 μl water. qRT-PCR was performed according to manufacturer’s protocol on Applied Biosystems—ABI PRISM 7700 instrument (Applied Biosystems, Foster City, CA, USA). To calculate the amount of gene-specific transcript, a standard curve was plotted for each primer set using a series of diluted genomic DNA from WT F. novicida. The amounts of each transcript were normalized to helicase rates (FTN_1594).

**Chromatin immunoprecipitation-qPCR assay.** Chromatin immunoprecipitation (ChIP) was performed with wild-type F. novicida (WT) and a Δfba strain expressing a His-tagged version of FBA (FBA-HA) (Fba/cpFBA-HA) bearing the 6x-His epitope fused at the C-terminal end of the protein. Bacteria were grown at 37°C in 100 mL TSB supplemented to mid-log (OD₆₀₀nm 0.3–0.4). Then bacteria were incubated in 24-well plates, in 1 mL of fresh medium for 1 h. In parallel, the bacterial suspension was divided by 100-fold excess of formaldehyde (Sigma) was added to a final concentration of 1% formaldehyde (Sigma) for 30 min, after which glycine (Sigma) was added to a final concentration of 125 fold excess of unlabeled probe. The labeled probe was incubated for 30 min:

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Amplification reactions were performed using Phusion High-Fidelity DNA Polymerase (ThermoScientific). The amplified fragments were purified using the primer pairs used in the EMSA and input samples) were divided by the following reactions:

**β-galactosidase assays.** Reporter fusion construction: Two lacZ transcriptional fusions were constructed by cloning either pkatg or prpoA promoter region upstream of the E. coli lacZ gene. The pkatg and prpoA promoter regions were amplified using the primer pairs used in the EMSA and E. coli 5171-xsp was the source of chromosomal DNA for amplification of the native lacZ gene. A 2075 bp region encompassing the entire coding sequence and its preceding Shine-Dalgarno sequence was amplified with the pair of primers: Fw, 5'-TATAATT-TAAAAGGAGGAGAAGCATT-3' and Rv, 5'-TTAAAAACGACGCCTAGT-3'. The labeled probe was incubated for 30 min; without FBA-HA, in the presence of FBA-HA, or in the presence of a 2-fold excess of unlabeled probe.

**Bacteria and cell culture infections.** J774.1 (ATCC TIB-67) cells were propagated in DMEM (PAA), containing 10% fetal bovine serum (FBS, PAA) unless otherwise stated. BMM from 6 to 8-week-old female BALB/c mice were grown in Roswell Park Memorial Institute (RPMI-1640) or DMEM, containing 10% FBS (PAA), respectively. RPMI-1640 was supplemented with 10% FBS (PAA) and 1% penicillin-streptomycin (100 U/mL). Bacteria were incubated in 1 mL of Z buffer with β-mercaptoethanol (60 mM NaHPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄ (pH 7.0), and 50 mM Na₂EDTA, pH 7.0 (Titriplex)). SDS and 0.1% BSA were added and the samples were vigorously vortexed 15 sec and kept 5 min at room temperature. Then, 200 μL of ONPG (4 mg mL⁻¹ in Z Buffer) were added and the samples were incubated at 30°C. The reactions were stopped by the addition of 1 M Na₂CO₃. The absorbance at 420 nm and 550 nm was measured and the data were converted in Miller units, using the classical formula \[
\text{OD}_{420} - \text{OD}_{550} \times (\text{Volume} \times \text{Time}) \times 1000
\]

**Cell culture and cell infection experiments.** J774.1 and Δkatg (Δkatg/OmpC) BMM were well seeded in 12-well cell tissue plates (in appropriate cellular culture medium supplemented with the appropriate carbon source) and bacterial strains were grown overnight in 5 mL of TSB at 37°C.

Infections were realized at a multiplicity of infection (MOI) of 100 and incubated for 1 h at 37°C in culture medium supplemented with the appropriate carbon source (glucose, glycogen, or pyruvate). We used in our assays DMEM without glucose supplemented either with glucose at 5 mM or with other carbon sources at the same molarity. After washes with cellular culture medium, plates were incubated for 4, 10, and 24 h in fresh medium supplemented with gentamicin (10 μg/mL). At each kinetic point, cells were washed 3 times with culture medium and lysed by addition of 1 mL of distilled water for 10 min at 4°C. The titers of viable bacteria was determined by spreadings on plates. Each experiment was conducted at least twice in triplicates.

**6-Production.** Supernatants from J774.1 infected with either wild-type F. novicida (WT) or Δfba mutant (MOI of 100) were harvested at 24 h. NI cells were tested as negative control. Cytokine were quantified by ELISA (BD) as previously described, using commercially available anti-IL-6 antibody in accordance with the manufacturer’s instructions.

**ROS detection assay.** Intracellular ROS were detected by using the oxidation-sensitive fluorescent probe dye,DCFDA as recommended by the manufacturer (DCFDA Cellular ROS Detection Assay Kit, Abcam, Cambridge, UK). J774.1 cells were seeded at 4 × 10⁴ cells per well. Cells were infected with bacteria for 10 or 24 h and incubated with DCFDA diluted in PBS (15 μM). DCF fluorescence was measured with a multiplate reader Berthold Tristar (Berthold France SAS, Thoire, France) with the use of excitation and
emission wavelengths of 480 and 525 nm, respectively. Values were normalized by protein concentration in each well (Bradford). Samples were tested in triplicates in two experiments.

**Determination of ROS generation via fluorescence microscopy.** J774.1 cells were seeded at 4×10^4 cells per well. Cells were infected with bacteria for 10 h (MOI of 1000:1), washed three times with PBS and incubated with DCFDA diluted in PBS for 1 h (15 μM). Images of the cells were captured with an Olympus CKX41 microscope and treated with Image J software. Cell counts were performed over 5 images of approx. 50 cells.

**Confocal experiments.** J774.1 macrophage cells were infected (MOI of 1,000) with wild-type *F. novicida* U112 (WT), the Δβfu isogenic mutant (Δβfu), or an isogenic strain deleted for the “Franciscella Pathogenicity Island” (Δβfu + ΔFP) in standard DMEM (DMEM-glucose) or DMEM without glucose and supplemented with either glucose or glyceral (5 mM each) for 30 min at 37 °C. Cells were then washed three times with PBS and maintained in fresh DMEM supplemented with gentamycin (100 μg/ml) before being treated with Image J software. Cell counts were performed over 5 images of approx. 50 cells.

**Proteomic analyses.** Protein digestion: FASP (Filter-aided sample preparation). Samples were reduced with 0.1 M dithiotreitol (DTT) for 30 min at 60 °C, then applied to the C18, Thermo Scientific filter units (Microcon, Millipore, Cat No MRCF0R030). The actual number of contaminant hits were removed from MaxQuant output. Proteins were quantified according to the MaxQuant label-free algorithm using LFQ intensities; protein quantification was obtained using at least 2 peptides per protein.

**Metabolomic analyses.** Central metabolite profiling by LC-MS/MS: Metabolome of bone marrow-derived macrophages cells was performed in NI condition, in cells infected either with the wild-type *F. novicida* strain (WT) or with a ΔFPi isogenic mutant. Central metabolites of cells were harvested and quantified 1 and 24 h after infection. After measurement of cultivation medium by aspiration, adherent cells were washed with 3 ml of PBS buffer, also eliminated by aspiration. Cells were then rapidly cultured in quenched plates with liquid nitrogen at −196 °C and extracted with 5 ml of a solvent mixture of ACN/Methanol/H₂O (2:2:1) at −20 °C. Samples were then evaporated and resuspended in 120 μl of ultrapure H₂O before analysis. The metabolites quantified were: Glucose-1-P (G1P), Glucose-6-P (G6P), UDP-glucose (UDP-Glc) Fructose-6-P (F6P), Fructose-Bis-P (FBP), Pentoses-5-P = Ribose-5P + Ribulose-5P + Xylulose-5P (SP5), Ribose-1-P (R1P), Sedoheptulose-7-P (SH7P), glyceral-3P (gly-3P), and 3-PhosphoGlycerate (3PG - PG), Phospho-Enol-Pyruvate (PEP), Citrate (Cit), ciss-aconitate (cis-aco), Oxoglutarate (OG), Succinate (Suc), Malate (Mal), Adenosine Mono, Di- and Tri-phosphate (AMP, ADP, ATP), Cytidine Mono, Di- and Tri-phosphate (CMP, CDP, CTP), Uridine Mono, Di- and Tri-phosphate (UMP, UDP,UTP), Guanidine Mono and Di-phosphate (GMP, GDP), UDP-Acetylgalacose (UDP-AcGluN), Shikimate-3-phosphate (Shikimate-3P), and Phosphor-Serine (P-Serine).

**Data availability.** The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD006908. The authors declare that all other data supporting the findings of this study are available within the paper and its Supplementary Information files.

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References

1. Sjøstedt, A. Tularemia: history, epidemiology, pathogen physiology, and clinical manifestations. Ann. N. Y. Acad. Sci. 1105, 1–29 (2007).

2. Santic, M., Molmeret, M., Klose, K. E. & Abu Kwaik, Y. Francisella tularensis travels a novel, twisted road within macrophages. Trends Microbiol. 14, 37–44 (2006).

3. McLendon, M. K., Apicella, M. A. & Allen, L. A. Francisella tularensis: taxonomy, genetics, and pathogenicity of a potential agent of biowarfare. Annu. Rev. Microbiol. 60, 167–185 (2006).

4. Kingry, L. C. & Petersen, J. M. Comparative review of intracellular bacterial pathogens and their mammalian host cells during intracellullar bacterial pathogens and their Mammalian host cells during infection ("pathotabemolism"). Microbiol. Spectr. 3, M3P-0002-2014 (2015).

5. Eshraghi, A. et al. Secreted effectors encoded within and outside of the Legionella pneumophila S4 DsbA-like FipB protein. (2016).

6. Ramsey, K. M. et al. Ubiquitous promoter-localization of essential virulence regulators in Francisella tularensis and Francisella novicida. Front. Cell. Infect. Microbiol. 4, 35 (2014).

7. Qin, A. et al. Components of the type six secretion system are substrates of Legionella pneumophila. FEMS Lett. 590, 3868–3886 (2016).

8. Beher, K., Eshraghi, A. & Petersen, J. M. Comparative review of intracellular bacterial pathogens and their Mammalian host cells during infection ("pathotabemolism"). Microbiol. Spectr. 3, M3P-0002-2014 (2015).

9. Eisele, N. A. et al. Salmonella require the fatty acid regulator PPARdelta for the intracellular metabolism of live tularemia vaccine prepared in a chemically defined medium. Appl. Microbiol. 13, 232–235 (1965).

10. Charity, J. C., Blalock, L. T., Costante-Hamm, M. M., Kasper, D. L. & Dove, S. M. Small molecule control of virulence gene expression in Francisella tularensis catalase restricts immune function by impairing TRPM2 channel activity. J. Biol. Chem. 291, 3871–3881 (2016).

11. Scudiero, D. A. et al. Cell death therapy ameliorates autoimmune disease through ablation of IL-6-producing B cells. J. Exp. Med. 209, 1001–1010 (2012).

12. Dieppedale, J. et al. Possible links between stress defense and the tricarboxylic acid (TCA) cycle in Francisella pathogenesis. Mol. Cell. Proteomics 12, 2278–2292 (2013).

13. Blume, M. et al. A toxoplasma gondii glycolytic enzyme contributes to Robust central carbon metabolism and is essential for replication and virulence. Cell Host Microbe 18, 210–220 (2015).

14. Shakerley, N. L., Chandrasekaran, A., Trebak, M., Miller, B. A. & Melendez, J. A. Francisella tularensis catalase restricts immune function by impairing TRPM2 channel activity. J. Biol. Chem. 291, 3871–3881 (2016).

15. Wehrly, T. D. et al. Intracellular biology and virulence determinants of Francisella tularensis revealed by transcriptional profiling inside macrophages. Cell. Microbiol. 11, 1128–1150 (2009).

16. Upchurch, R. T. et al. Intracellular biology and virulence determinants of Francisella tularensis revealed by transcriptional profiling inside macrophages. Cell. Microbiol. 11, 1128–1150 (2009).

17. Raghunathan, A., Shao, X. & Daehler, S. Systems approach to investigating host-pathogen interactions in infections with the biothreat agent Francisella. Constraints-based model of Francisella tularensis. BMC. Syst. Biol. 4, 118 (2010).

18. Steele, S. et al. Francisella tularensis harvests nutrients derived via ATG5-independent autophagy to support intracellular growth. PLoS Pathog. 9, e1003562 (2013).

19. Chico-Calero, I. et al. Hpt: the bacterial homolog of the micromosaic glucose-6-phosphate translocase, mediates rapid intracellular proliferation in Listeria. Proc. Natl Acad. Sci. USA 99, 431–436 (2002).

20. Wright, R. H. et al. ADP-ribose-derived nuclear ATP synthesis by NUDIX5 is required for chromatin remodeling. PLoS ONE 7, e52378 (2012).

21. Meireles Dde, A., Alegría, T. G., Alves, S. V., Arantes, C. R. & Netto, L. E. A 14.7 kDa protein from Francisella tularensis subs. novica (named FTN_1133), involved in the response to oxidative stress induced by organic peroxides, is not endowed with thiol-dependent peroxidase activity. PLoS ONE 9, e99492 (2014).

22. Ritterson Lew, C. & Tolan, D. R. Aldolase sequencers WASP and affects WASP/Arp2/3-stimulated actin dynamics. J. Cell. Biochem. 114, 1928–1939 (2013).

23. Alefounder, P. R. & Perham, R. N. Identification, molecular cloning, and sequence analysis of a gene cluster encoding the class II fructose 1,6-biphosphatase aldolase, 3-phosphoglycerate kinase and a putative second glycolysis-3-phosphate dehydrogenase of Escherichia coli. Mol. Microbiol. 3, 723–732 (1989).

24. Thomson, G. J., Howlett, G. G., Ashcroft, A. E. & Berry, A. The dhaA gene of Escherichia coli encodes a class I fructose bisphosphatase aldolase. Biochem. J. 331 (Pt 2), 437–445 (1998).

25. Plaumann, M., Pelzer, R., Börner, M., W.F. & Schnaasferber, C. Multiple recruitment of class-I aldolase to chloroplasts and eubacterial origin of eukaryotic class-II aldolases revealed by cDNAs from Euglena gracilis. Curr. Genet. 31, 430–438 (1997).

26. de la Paz Sanlagelo, M. et al. Glycolytic and non-glycolytic functions of Mycobacterium tuberculosis fructose-1,6-bisphosphatase aldolase, an essential enzyme produced by replicating and non-replicating bacilli. J. Biol. Chem. 286, 40219–40231 (2011).
55. Knowles, H., Li, Y. & Perraud, A. L. The TRPM2 ion channel, an oxidative stress and metabolic sensor regulating innate immunity and inflammation. *ImmunoL Res.* 55, 241–248 (2013).

56. Knowles, H. et al. Transient receptor potential melastatin 2 (TRPM2) ion channel is required for innate immunity against *Listeria monocytogenes*. *Proc. Natl Acad. Sci. USA* 108, 11578–11583 (2011).

57. Lee, B. Y., Horwitz, M. A. & Clemens, D. L. Identification, recombinant expression, immunolocalization in macrophages, and T-cell responsiveness of the major extracellular proteins of *Francisella tularensis*. *Infect. Immun.* 74, 4002–4003 (2006).

58. McCaig, W. D., Koller, A. & Thanassi, D. G. Production of outer membrane vesicles and outer membrane tubes by *Francisella novicida*. *J. Bacteriol.* 195, 1120–1132 (2013).

59. Bandyopadhyay, S., Long, M. E. & Allen, L. A. Differential expression of microRNAs in *Francisella tularensis*-infected human macrophages: miR-155-dependent downregulation of MyD88 inhibits the inflammatory response. *PLoS ONE* 9, e109525 (2014).

60. Broz, P. & Monack, D. M. Molecular mechanisms of inflammasome activation during microbial infections. *Immunol. Rev.* 243, 174–190 (2011).

61. Henderson, B. An overview of protein moonlighting in bacterial infection. *Biochem. Soc. Trans.* 42, 1720–1727 (2014).

62. Tunio, S. A. et al. The moonlighting protein fructose-1, 6-bisphosphate aldolase of *Neisseria meningitidis*: surface localization and role in host cell adhesion. *Mol. Microbiol.* 76, 605–615 (2010).

63. Shams, F. et al. Fructose-1,6-bisphosphate aldolase of *Neisseria meningitidis* binds human plasminogen via its C-terminal lysine residue. *Microbiol. Open* 5, 340–350 (2016).

64. Flores-Ramirez, G., Jankovicova, B., Bilkova, Z., Miernyk, J. A. & Skultety, L. Identification of *Coxiella burnetii* surface-exposed and cell envelope associated proteins using a combined bioinformatics plus proteomics strategy. *Proteomics* 14, 1868–1881 (2014).

65. Loughman, J. A. & Caparon, M. Regulation of Spcll in Streptococcus pyogenes by pH and NaCl: a model for in vivo gene expression. *J. Bacteriol.* 188, 399–408 (2006).

66. Ciesla, M., Mierzejewska, J., Adamczyk, M., Farrants, A. K. & Boguta, M. Fructose bisphosphate aldolase is involved in the control of RNA polymerase III-directed transcription. *Biochim. Biophys. Acta* 1843, 1103–1110 (2014).

67. Lincet, H. & Icard, P. How do glycolytic enzymes favour cancer cell proliferation by nonmetabolic functions? *Oncogene* 34, 3751–3759 (2015).

68. Ishihama, A. Role of the RNA polymerase alpha subunit in transcription activation. *Mol. Microbiol.* 6, 3283–3288 (1992).

69. Anthony, L. D., Burke, R. D. & Nano, F. E. Growth of *Francisella* spp. in rodent macrophages. *Infect. Immun.* 59, 3291–3296 (1991).

70. Lipecka, J. et al. Sensitivity of mass spectrometry analysis depends on the shape of the filtration unit used for filter aided sample preparation (FASP). *Proteomics* 16, 1852–1857 (2016).

71. Cox, J. & Mann, M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat. Biotechnol.* 26, 1367–1372 (2008).

72. Cox, J. et al. Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ. *Mol. Cell. Proteomics* 13, 2513–2526 (2014).

73. Tyanova, S. et al. The perseus computational platform for comprehensive analysis of (pro)teomics data. *Nat. Methods* 13, 731–740 (2016).

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**Author contributions**

J.Z. performed most of the in vitro experiments; F.T. performed murine in vivo experiments and some immunofluorescence experiments; I.C.G. and C.C. performed the proteomic analyses and I.C.G. analyzed and compiled the data; E.C. and L.G. performed metabolomic analyses and E.C. analyzed and compiled the data; M.A., M.D., and S.K. performed some in vitro experiments; M.B. analyzed the data; A.C. designed the experiments, and J.Z. and A.C. analyzed the data and wrote the paper. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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