SUPPORTING INFORMATION

Supplemental Information includes six Figures, two Tables, Supplemental Experimental Procedures, and Supplemental References.

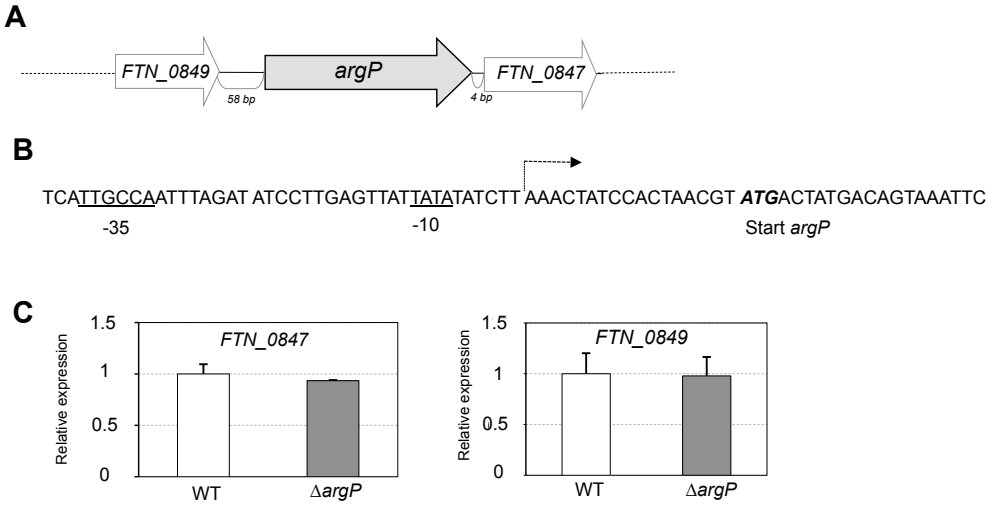

Figure S1. Sequence analysis of *argP* region

(A) Schematic organization of the region. The gene *argP* (*FTN_0848*) encoding ArgP APC superfamily transporter is colored in grey. It is flanked, upstream (58 bp) by gene *FTN_0849* and downstream (4 bp) by gene *FTN_0847*, both are transcribed on the same strand.

(B) Transcriptional analysis. We realized rapid amplification of cDNA ends (5' race) to determine the 5'end of the *argP* mRNA. A broken arrow shows the transcription start of *argP* (+1). Inspection of the sequence immediately upstream of the transcriptional start identified putative -10 and -35 promoter elements that share homology to the consensus site recognized by the major sigma factor σ^{70}. The predicted σ^{70}-dependent -10 and -35 sequences are underlined. The predicted translation start codon of *argP* is in bold italics.

(C) Quantitative real-time RT-PCR. Quantification of *FTN_0847* and *FTN_0849* expression in wild-strain *F. novicida* and Δ*argP* mutant, were performed in TSB at 37°C. Analyses were performed twice using independent samples (in triplicates).

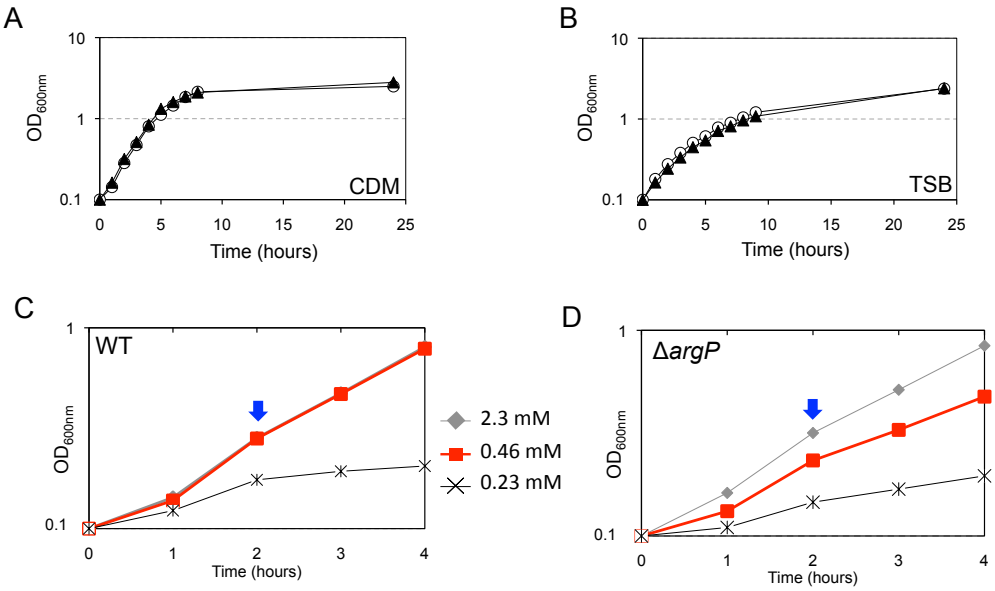

Figure S2. Bacterial growth in broth

Bacterial growth was monitored at OD_{600nm} over a 24 h-period, **(A)** in standard Chemically defined medium (CDM); **(B)** in Tryptic soya broth (TSB). Open circles, wild-type *F. novicida*; black triangles, Δ*argP* mutant.

Effect of different arginine concentrations on wild-type *F. novicida* (**C**) and Δ*argP* mutant strain (**D**) growth was monitored over a 5 h-period. Growth was evaluated in CDM with 2.3 mM arginine, 0.46 mM arginine and 0.23 mM arginine. The blue arrows indicate the time point taken for the Mass spectrometry analyses.

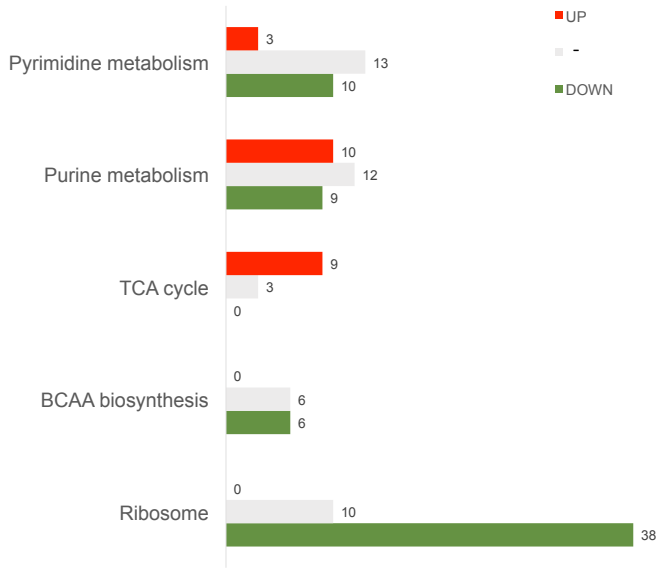

Figure S3. KEGG pathways

The selected 842 proteins belong to multiple pathways, according to the KEGG pathway database for *F. novicida* (available at http://www.genome.jp/kegg-bin/show_organism?menu_type=pathway_maps&org =ftn). This histogram depicts the distribution of proteins belonging to five different pathways (Pyrimidine or purine metabolism, tricarboxylic acid (TCA) cycle, branched-chain amino acid bisosynthesis, ribosomal proteins.

Red bars (Up), proteins present in higher amounts in the Δ*argP* mutant.

Green bars (Down), proteins present in lower amounts in the Δ*argP* mutant.

Gray bars (-), unchanged amounts.

Numbers on top of each bar indicate the total number of proteins.

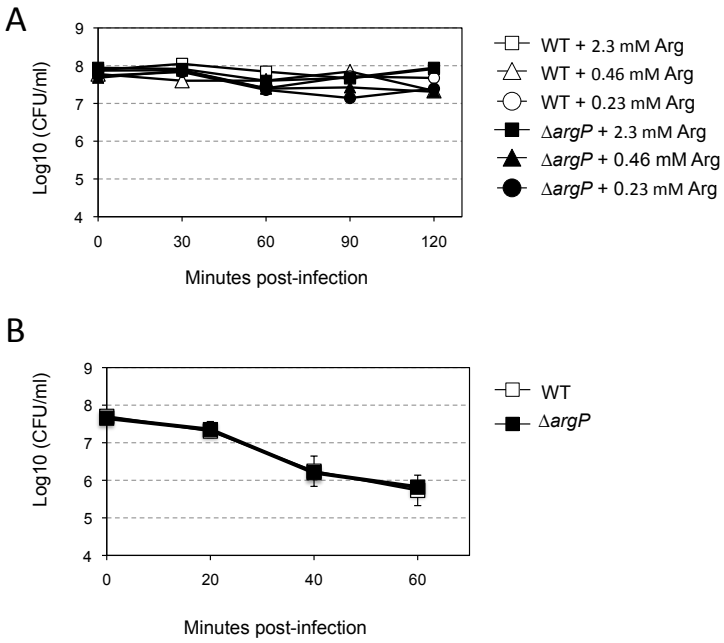

Figure S4. Sensitivity to pH and oxidative stresses

(A) pH stress. Bacteria were incubated for 2 h in CDM at pH 4, the presence of various concentrations of arginine. *i.e*. 2.3 mM (corresponding to the arginine concentration present in standard CDM), 0.46 mM and 0.23 mM, respectively. At 30 min intervals, bacteria were collected and numerated on TSB solid medium.

(**B**) **Oxidative stress.** Exponential phase bacteria, diluted in TSB medium were subjected to oxidative stress (500 μM H_20_2). The bacteria were plated on chocolate agar plates at different times and viable bacteria were monitored 2 days after. Data are the average c.f.u mL^{-1} for three points. Experiments were realized twice.

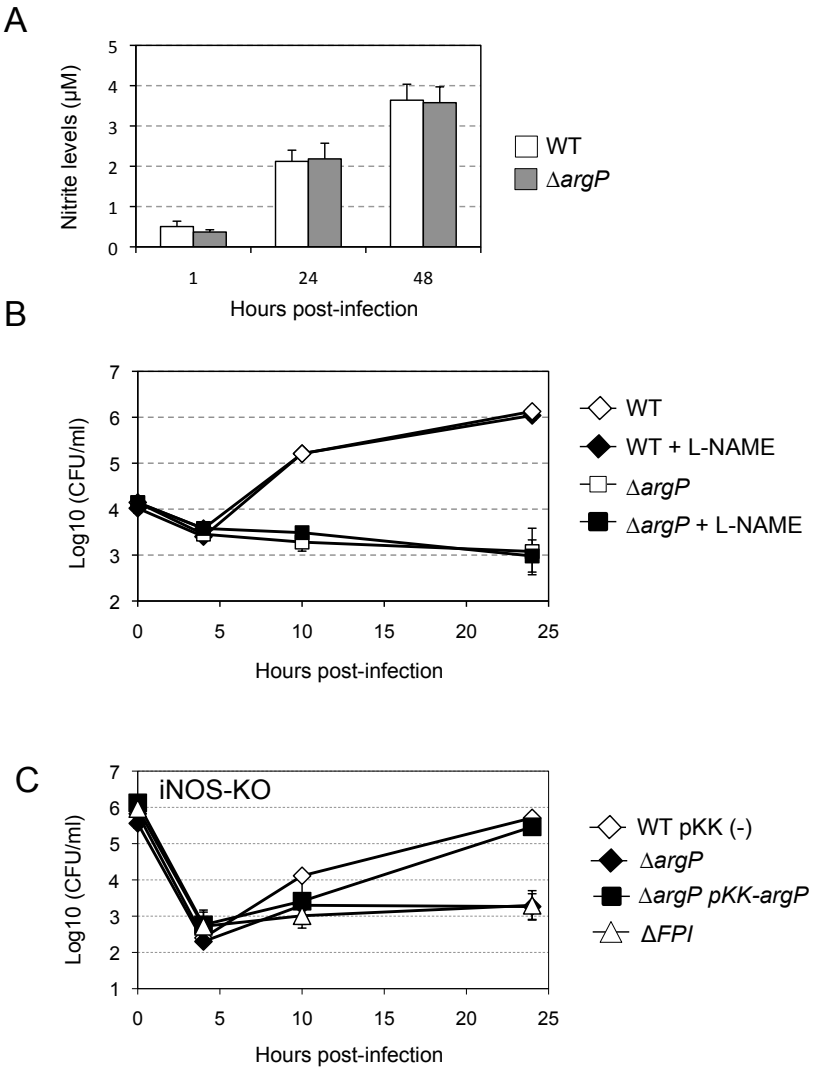

Figure S5. Susceptibility to NO

(A) Nitrite levels. Nitric oxide amount was determined by the Griess test in J774.1 cells infected with Wild-type *F. novicida* and Δ*argP* mutant for 1 h. Supernatants were analyzed after 1 h, 24 h and 48 h of infection. Data were normalized with non-infected cells nitrites amount. They are expressed as means ± standard deviations for three determinations. Experience was realized twice.

(**B**) **Intracellular multiplication in the presence of the arginine analog L-NAME.** J774.1 cells were treated with 1 mM iNOS inhibitor L-NAME before and all along

the infection with Wild-type *F. novicida* and Δ*argP* mutant. At several time-points, cells were washed and lysed by the addition of demineralized water. For each strain and time in an experiment, the assay was performed in triplicate. Each experiment was independently repeated twice and the data presented originate from one typical experiment.

(**C**) **Intracellular multiplication in iNOS-KO BMM.** Representative intracellular multiplication of wild-type *F. novicida* (WT) carrying the empty plasmid pKK214 (WT pKK (-)), of the Δ*argP* mutant (Δ*argP*) and complemented strain (Δ*argP* pKK-*argP*), and of the Δ*FPI* mutant (Δ*FPI*), in iNOS-deficient BMMs over a 24 h period. Results are shown as the average of $\log_{10}$ (CFU/ml) ± standard deviations. Experiences were realized twice.

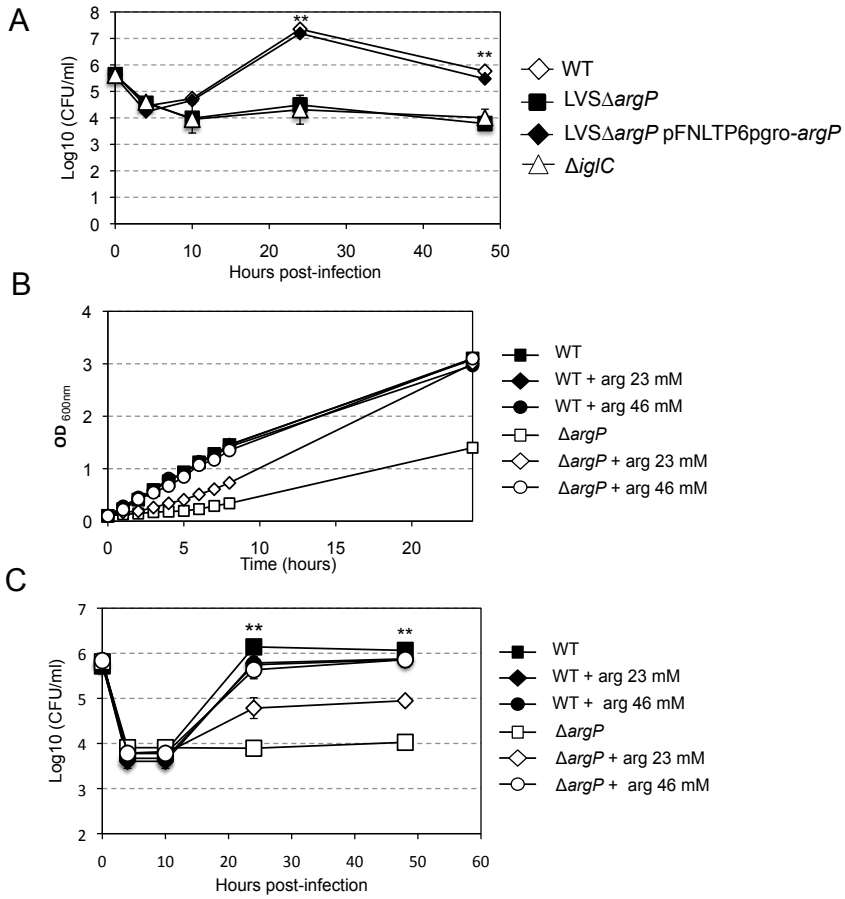

Figure S6. Critical role of ArgP in *F. tularensis* LVS

(A) Intracellular replication in J774.1 cells. The LVSΔ*argP* mutant showed a severe intracellular replication defect in J774.1 cells, in standard DMEM. Wild-type intracellular multiplication was restored in the complemented strain. Each experiment was performed in triplicate. **P < 0.01 (as determined by Student's t -test).

(B) Growth in synthetic medium. Growth of wild-type LVS and LVSΔ*argP* mutant was studied in CDM supplemented with various concentrations of arginine. In standard CDM, LVSΔ*argP* mutant showed a strong growth defect. This defect was partially alleviated upon supplementation with 23 mM arginine and wild-type growth was restored upon supplementation with 46 mM arginine Experiments were repeated twice. The figure represents a typical experiment.

(C) Arginine supplementation restores LVSΔ*argP* intracellular multiplication. Standard DMEM was supplemented with either 26 mM or 43 mM arginine. At 43 mM arginine, the multiplication of LVSΔ*argP* was restored to wild-type levels in J774.1 cells. Each experiment was performed in triplicate. **P < 0.01 (as determined by Student's t -test).

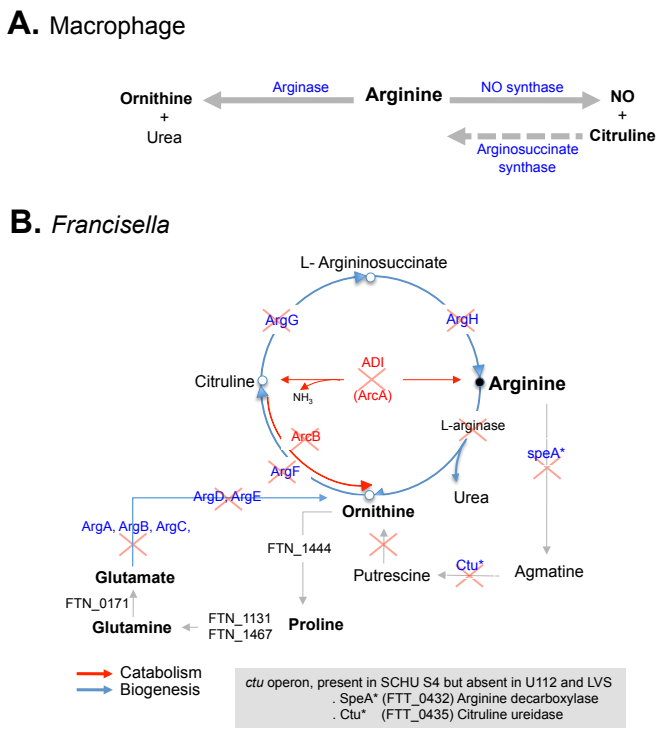

Figure S7. Arginine metabolic pathways

(**A**) Overview of mammalian arginine metabolism. Arginase and NOS use arginine as a common substrate and compete with each other for this substrate.

(**B**) In *F. novicida*, all the genes for the biosynthesis or degradation of arginine are inactive or altered (according to KEGG metabolic pathways; symbolized by a red cross). Grey arrows symbolize the predicted enzymatic reactions.

Table S1. Detailed list of proteins quantified by mass spectrometry submitted to statistical analysis (see attached xlsx file)

Table S2. Strains, plasmids and primers

Strain, plasmid, or primer	Primer code	Description or sequence (5' → 3')	Reference or source
E. coli strains			
E. coli Top10		F– *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1*ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Laboratory strain collection
F. tularensis strains			
U112		*F. tularensis* subsp. *novicida* U112	Laboratory strain collection
U112Δ*FPI*		Chromosomal deletion of *the entire Francisella pathogenicity island (FPI)*	(Weiss et al., 2007)
FTN (PKK214)		FTN containing empty plasmid PKK214	This study
FTNΔ*FTN_0848*		FTN with gene *FTN_0848* deleted	This study
FTNΔ*FTN_0848* (PKK214-*FTN_0848)*		FTNΔ*FTN_0848* containing PKK214-*FTN_0848*	This study
LVS		*F. tularensis* ssp *holarctica* strain LVS	A. Sjostedt
LVSΔ*FTL_1233*		Chromosomal deletion of *FTL_1233*	This work
LVS Δ*FTL_1233* (pFNLTP6p*gro-FTL_1233*)		LVSΔ*FTN_1233* containing complementing plasmid pFNLTP6 p*gro-FTL_1233*	This work
LVSΔiglC		Chromosomal deletion of *iglC*	A. Sjostedt
Plasmids			
pGEM		*E. Coli* cloning vector, Amp^R	Promega
pGEM-*FTL_1233* up/down		pGEM containing the indel; Amp^R	This work
pKK214		Derived from pKK202, promoter trap vector drives Cm^R Tet^R	(Kuoppa et al., 2001)
pKK214-*FTN_0848*		pKK214 containing the gene *FTN_0848*	This work
pMP812		*sacB* suicide vector, Kan^R	(LoVullo et al., 2009)
pMP812-*FTL_1233* up/down		pMP812 containing the indel; Kan^R	This work
pFNLTP6 p*gro*		*E. coli / F. tularensis shuttle vector; Kan^R Amp^R* Expression of the kanamycine resistance *npt* gene under p*gro* promoter control	(Maier et al., 2006)
pFNLTP6 p*gro-FTL_1233*		pFNLTP6 p*gro* containing the gene *FTL_1233*	This work
Primers			
Pgro_F	1	TTG TAT GGA TTA GTC GAG CTA AA	
npt_R	2	TCA GAA GAA CTC GTC AAG AAG G	
FTN_0848upF	3	CCC AAG TGC AGA AAA ATG TGA G	
FTN_0848upR	4	GAG CTT TTT AGC TCG ACT AAT CCA TAC AAC ATA CGT TAG TGG ATA GTT TAA G	
FTN_0848downF	5		
FTN_0848downR	6		
FTN_0848upF2 control	7	AGG TAT AAA CTA TGC AAG TAT C	
FTN_0848downR2 control	8	TTT TTG AAG AGA TCT TTG AG	
FTN_0848 5'race GSP1	9	TCA AAA TAC TTG GAA ACC AGA TAA G	
FTN_0848 5'race GSP2	10	TAT CCA TTG GAA CCA TAC AGC	
FTN_0848 compl forw	11	CCC GGG ATC AAG ATA GAT TAA TCA TCA AAG TGG	
FTN_0848 compl rev	12	GAA TTC AAC AAG AGT TAG AAA AAG TAT TAA TGA TT	
FTN_0847 qRT forw	15	CTC TCA AAA TTG TAC GGT TGA TAA ATC G	
FTN_0847 qRT rev	16	CAG TAT AAC CGA CAC TTA TAC CGA TAA CTT C	
FTN_0848 qRT forw	13	CTT CCA TCT ACA GCC ACT TCA GGA	
FTN_0848 qRT rev	14	GCA GTA TAT GTC GTA GTC ATC TCT GCA	
FTN_0849 qRT forw	17	CTG TTA TTG ATC TAG ATG AAG AAA AAC TTG G	
FTN_0849 qRT rev	18	ATT TGA TGT CAC AGC TAT TAT CAT ATC AGT ATC	
FTL_1233 upF NotI	19	ATG TAG CGG CCG CCC TTT CCG AAA AAAG C	
FTL_1233 upR	20	ACA AAT TAT CTG GTG GGA CAA AGC CGT T	
FTL_1233 doF	21	TTC CAT CTA CAG CCA CTT CAG GAA CGG CT	
FTL_1233 doR BamHI	22	CCA TAG GAT CCG CAG TAT TAG CGG CAT A	

Supplemental Experimental procedures

Bacterial growth

F. novicida and its mutant derivatives were grown: i) in liquid, in Tryptic Soya broth (Becton, Dickinson and company) or Chamberlain chemically defined medium and ii) in solid, on pre-made chocolate agar PolyViteX (BioMerieux SA Marcy l'Etoile, France) or chocolate plates prepared from GC medium base, IsoVitalex vitamins and haemoglobin (BD Biosciences, San Jose, CA, USA), at 37°C. *E. coli* was grown in LB (Luria-Bertani, Difco) at 37°C. Ampicillin was used at a final concentration of 100 μg mL^{-1} to select recombinant *E. coli* carrying pGEM and its derivatives. Kanamycin was used at a final concentration of 50 μg mL^{-1} and 15 μg mL^{-1} to select respectively recombinant *E. coli* and *Francisella* carrying pKK and its derivates.

All bacterial strains, plasmids, and primers used in this study are listed in **Table S1**.

Construction of chromosomal Δ*argP* deletion mutants

In *F. novicida*. We have generated a chromosomal deletion of gene *FTN_0848* (*argP*) in *F. novicida* strain U112 by allelic replacement of the wild-type region with a mutated region deleted of the entire *argP* gene (from the ATG start codon till the TAA stop codon), substituted by the kanamycine resistance gene *npt* placed under the control of the P*gro* promoter. First, the two regions (app. 500 bp each) flanking gene *argP* (designated *FTN_0848*up and *FTN_0848*down, respectively), and the *npt* gene (1,161 bp, amplified from plasmid pFNLTP16H3 (Maier et al., 2006), were amplified by PCR using the following pairs of primers: i) *FTN_0848*up, p3, p4; ii) *FTN_0848*down, p5, p6; iii) *npt*, p1, p2. The region *FTN_0848*up-*npt*-

*FTN_0848*down (*ca.* 2300 bp) was then amplified by triple overlap PCR, using the *FTN_0848*up, *FTN_0848*down and *npt* products. The resulting PCR product was gel purified (using the QIAquick Gel extraction kit, QIAgen) and directly used to transform wild-type *F. novicida*. Chemical transformation was performed as described previously (Ludu et al., 2008). Recombinant bacteria, resulting from allelic replacement of the wild-type region with the mutated *FTN_0848up/npt/ FTN_0848down* region, were selected on kanamycine-containing plates (15 µg mL^{-1}). The mutant strain, designated Δa*rgP,* was checked for loss of the wild-type *argP* gene, using specific primers in PCR and qRT-PCR, by PCR sequencing (GATC Biotech) and Southern blot.

In *F. tularensis* LVS. We generated a chromosomal deletion of the orthologous gene *FTL_1233* in *F. tularensis* LVS, by using the counter-selectable plasmid pMP812 (LoVullo et al., 2009). The recombinant plasmid pMP812-Δ*FTL_1645* was constructed by overlap PCR. Primers p15/p16 amplified the 994 bp region upstream of position + 1 of the *FTL_1645* coding sequence, and primers p17/p18 amplified the 1,012 bp region immediately downstream of the *FTL_1645* stop codon (Table S1). Primers p16/p17 have an overlapping sequence of 23 nucleotides, resulting in complete deletion of the *FTL_1233* coding sequence after cross-over PCR. PCR reactions with primers p15/p16 and p17/p18 were performed with exTaq polymerase (Fermentas). The products were purified using the QIAquick PCR purification kit (QIAgen, CA). 200 ng of each was used as a template for PCR with primers p15/p18 and treated with 30 cycles of PCR (94°C for 30 s, 54°C for 30 s and 72°C for 120 s). The gel-purified 1,996 bp fragment was digested with *BamH*I and *Not*I (New England Biolabs) and cloned into *BamH*I–*Not*I digested pMP812 (LoVullo et al., 2009). The plasmid is introduced into *F. tularensis* LVS by electroporation. *F. tularensi*s LVS

was grown to OD 600 0.3–0.6 in Schaedler-K13 broth; bacteria were collected and washed twice with 0.5 M sucrose. Bacteria were suspended in 0.6 mL of 0.5 M sucrose and 200 µL were used immediately for electroporation in a 0.2 cm cuvette (2.5 kV, 25 mF, 600 W). After electroporation, bacteria were mixed with 1 ml of Schaedler-K3 broth and incubated at 37°C for 6 h before selection on chocolate agar (Bio-Rad, Hercules, CA, USA) with 5 µg mL^{-1} kanamycine concentration. Colonies appeared after 3 days of incubation at 37°C and were subsequently passed once on plates with selection, followed by a passage in liquid medium without selection (to allow recombination to occur). Next, bacteria were passed once on agar plates containing 5% sucrose. Isolated colonies were checked for loss of the wild-type *FTL_1233* gene by size analysis of the fragment obtained after PCR using primers combination p15/p18, p33/p18 and p15/p34. One colony harbouring a *FTL_1645* deletion, as determined by PCR analysis, was used for further studies. Genomic DNA was isolated and used as the template in a PCR with primers p33/p34. The PCR product was directly sequenced using primers p33/p34 to verify the complete deletion of the *FTL_1233* gene.

Functional complementation

The plasmid used for complementation of the Δ*argP* mutant, pKK-*argP*, was constructed by amplifying a 1,612 bp fragment (corresponding to the sequence 92 bp upstream of the *argP* start codon and to 40 bp downstream of the stop codon) using primers *FTN_0848* compl forw and *FTN_0848* compl rev (**Table S2**), followed by digestion with *Eco*RI and *Sma*I, and cloning into plasmid pKK214 (Kuoppa et al., 2001).

The plasmids pKK214 and pKK214-*argP,* were introduced into wild-type *F. novicida*

by electroporation, as described previously (Dieppedale et al., 2011).

Multiplication in macrophages

J774.1 macrophage-like cells (ATCC® Number: TIB67™) were propagated in Dulbecco´s Modified Eagle's Medium (DMEM) containing 10% fetal calf serum. Bone marrow-derived macrophages (BMM) from BALB/c mice, iNOS-KO mice, C57Bl/6j mice and IRAP-KO C57Bl/6j mice, were differentiated in RPMI 1640 Medium containing 10% fetal calf serum and 10% L-CSF. Bone marrow-derived dendritic cells (BM-DC) from C57Bl/6j mice and IRAP-KO C57Bl/6j mice were differentiated in Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum and 3% GM-CSF. J774.1, BMM and BM-DC, were seeded at a concentration of ~2 x 10^5 cells per well in 12-well cell tissue plates and monolayers were used 24 h after seeding. All cell types were incubated for 60 min at 37°C with the bacterial suspensions (at MOI of 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 DMEM, RPMI or Iscove's modified Dulbecco's medium depending on the kind of phagocytic cells. Cells were lysed by addition of demineralized water and the titer of viable bacteria released from the cells was determined by spreading serial dilutions 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 twice and the data presented originate from one typical experiment.

Confocal experiments

J774.1 macrophage-like cells were seeded at 5.10^5 cells per well on glass coverslips in 12-well bottom flat plates. Next day, cells were infected at a MOI of 1,000 with wild-type *F. novicida*, Δ*argP* or Δ*FPI* strains for 1 h, 4 h and 10 h at 37°C. For each time point, cells were fixed with paraformaldehyde (4%) for 15 min, quenching was realized with NH_4Cl for 10 min and cells were permeabilized and blocked with 0.1% saponine, 5% goat serum in PBS for 10 min. All antibodies were diluted in the same mix and all steps are separated by PBS washing. Monolayers were then incubated with anti *F. tularensis* ssp *novicida* mouse monoclonal antibody (Creative diagnostics) diluted 1:500 and anti LAMP-1 rabbit monoclonal antibody (Abcam), diluted 1:250, for 30 min at room temperature (RT) followed with donkey anti-mouse Alexa Fluor 488 (Abcam) and donkey anti-rabbit Alexa fluor 546 (Abcam), both diluted 1:400, for 30 min. Monolayers were then incubated 1 min in DAPI diluted 1:1000 in PBS. Coverslips were washed in PBS and in demineralized water and then mounted in Mowiol.

Cells were examined using an X63 oil-immersion objective on a LeicaTSP SP5 confocal microscope. Co-localization tests were performed by using Image J software; and mean numbers were calculated on more than 500 cells for each condition. Confocal microscopy analyses were performed at the Cell Imaging Facility (Faculté de Médecine Necker Enfants-Malades).

Electron microscopy

J774 cells were infected with wild-type *F. novicida* and mutant Δ*ansP* bacteria. Samples for electron microscopy were prepared using the thin-sectioning procedure as previously described (Alkhuder et al., 2009).

Vacuolar rupture assay

Quantification of vacuolar escape by flow cytometry using the β -lactamase-CCF4 assay (Life technologies) was performed following manufacturer's instructions. Briefly, macrophages were infected in non-Tc treated plates with Wild-type *F. novicida*, Δ*argP* mutant or Δ*FPI* mutant with a MOI of 10. At 2 h post-infection (PI) , macrophages were incubated with CCF4 for 1 h at RT in the presence of 2.5 mM probenicid (Sigma). Cells were collected by gentle scraping, washed once, resuspended in PBS containing 2.5 mM probenicid and propidium iodide at 5 ug mL^{-1} and directly analysed by flow cytometry on a canto 2 cytometer (BD Bioscience). Propidium iodide negative cells were considered for the quantification of cells containing cytosolic *F. novicida* using excitation at 405 nm and detection at 450 nm (cleaved CCF4) or 535 nm (intact CCF4).

Real time cell death assay

Cell death was quantified in real time by monitoring propidium iodide incorporation. Briefly, 5.10^4 BMM were seeded in 0.3 cm^2 wells of black 96-flat-bottom-well plate and infected with Wild-type *F. novicida*, Δ*argP* mutant or Δ*FPI* mutant with an MOI of 100. Two hours post-infection, cells were washed and incubated in CO_2-independent medium (Gibco) containing 5 ug mL^{-1} propidium iodide, MCSF, 10% FCS and 2 mM glutamine. The 96 wells plate was immediately transferred to a microplate fluorimeter (Tecan infinite M1000) prewarmed at 37°C. Propidium iodide fluorescence was measured every 15 min over a 15 h-period.

Isolation of total RNA and reverse transcription

Bacteria were centrifuged for 2 min at RT and the pellet was quickly resuspended in Trizol solution (Invitrogen, Carlsbad, CA, USA). Samples were either processed immediately or frozen and stored at -80°C. 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 DNase digestion of 30 min (Thomson et al., 2008).

RNA Reverse transcription (RT)-PCR experiments were carried out with 500 ng of RNA and 2 pmol of specific reverse primers. After denaturation at 65°C for 5 min, 6 μL of the mixture containing 4 μL of 5X first strand buffer and 2 μL of 0.1 M DTT were added. Samples were incubated for 2 min at 42°C, then 1 μL of Superscript II RT (Thermo Scientific) was added. Samples were incubated for 50 min at 42°C, heated at 70°C for 15 min and chilled on ice. Samples were diluted with 180 μL of H_2O and stored at –20°C.

The following pair of primers was used to amplify the mRNA corresponding to the transcript of *FTN_0847* (p15/p16), *FTN_0848* (p13/p14), *FTN_0849* (p15/p16) (**Table S1**).

Quantitative real-time RT-PCR

The 25 μL reaction consisted of 5 μL of cDNA template, 12.5 μL of Fastart SYBR Green Master (Roche Diagnostics), 2 μL of each primer (at 10 μM) and 3.5 μL water. qRT-PCR was performed according manufacturer's protocol on Applied Biosystems - ABI PRISM 7700 instrument (Applied Biosystems, Foster City, CA). 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 wild-type *F. novicida*. The amounts of each transcript were normalized to helicase rates (*FTN_1594*).

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