Diverse roles of Low-Molecular Weight Thiol GSH in *Francisella*'s virulence, location sensing and GSH-stealing from host

Running Title: Role of GSH in *Francisella*

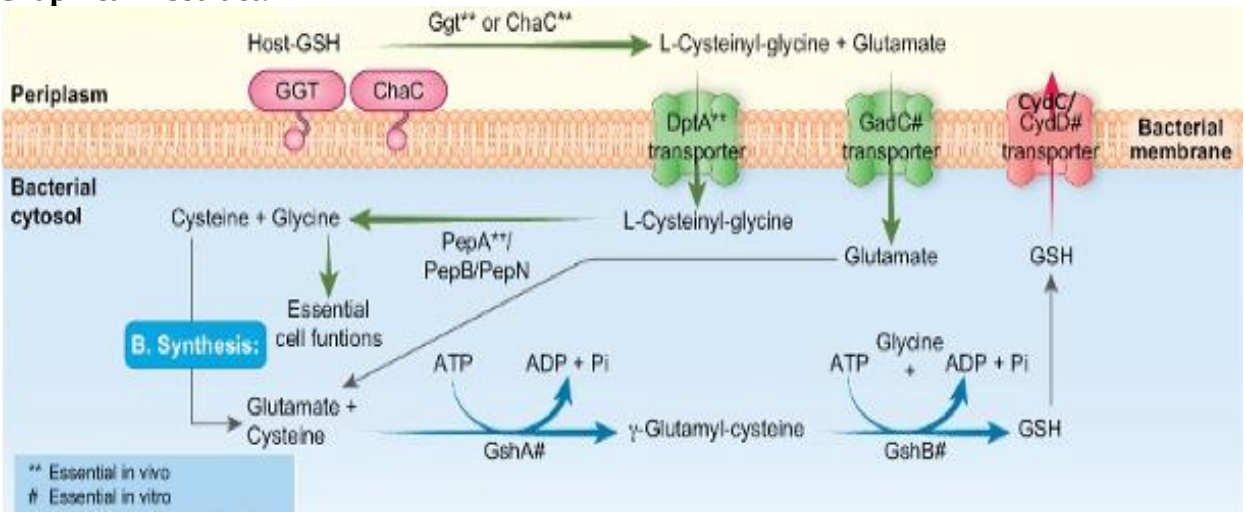
Monique van Hoek (*) [1], Alexander Marchesani [2], Mamta Rawat [3]
(*) Corresponding author, Mail Stop 1H8, 10910 George Mason Circle, George Mason University, Manassas, VA 20109, mvanhoek@gmu.edu

1. School of Systems Biology, George Mason University, Manassas, VA
2. Department of Biology, George Mason University, Manassas, VA.
3. Biology Department, California State University, Fresno, CA.

Abstract:

Low-molecular weight (LMW) thiols, encompassing peptides and small proteins with active cysteine residue(s), are important to bacteria as they are involved in a wide range of redox reactions. They include the tripeptide glutathione (GSH) and the small redox proteins, thioredoxins and glutaredoxins. We review the low MW thiols and related molecules in *Francisella* species and what role they may play in growth and virulence. Genes for GSH biosynthesis, metabolism and thioredoxins are present in all strains of *Francisella*, including the fully human-virulent strains. GSH and cysteine (CSH) are the major LMW thiols in in *Francisella* extracts. We explore the potential role of the LMW thiols to overcome the nutritional challenges of intracellular growth (high GSH conditions) as well as the nutritional challenges of planktonic growth (low GSH conditions), and their contribution to *Francisella*'s sensing its environmental location. *Francisella* may also use GSH as a source of CSH, for which it is auxotrophic. "Glutathione stealing" from the host may be an important part of *Francisella*'s success strategy as a facultative intracellular pathogen both to detect its location and obtain CSH. An understanding of GSH metabolism in *Francisella* provides insights into the interaction of this pathogen with its host and may reveal additional targets for therapeutic intervention for tularemia infections.

Graphical Abstract:



Keywords: Glutathione, GSH, bacterial GSH-cleaving enzymes, Glutamate importer, GSH exporter, Francisella, Thioredoxin, Glutraredoxin.

Abbreviations:
BHI, Brain Heart Infusion;
CHAB, CSH heart agar supplemented with 1% hemoglobin;
CSH, reduced CSH;
DNA, Deoxyribonucleic Acid;
GGCT (**γ–glutamyl-cyclotransferase**);
GGT, Gamma-Glutamyl Transpeptidase (GGT);
GSH, Glutathione, γ-L-Glutamyl-n-cysteinylglycine;
GSNO, *S*-nitrosoglutathione;
GSSG, oxidized GSH;
HPLC, High Performance Liquid Chromatography;
LMW, low molecular weight;
LVS, Live Vaccine Strain;
ROS, Reactive Oxygen Species;
TSBC, Trypticase soy broth with 0.1% CSH;

Introduction:

The role of low molecular weight (LMW) thiols and related molecules in pathogenesis and virulence of bacterial pathogens is slowly beginning to emerge. We were interested to examine what LMW thiols are used in *Francisella* species and what, if any, role in virulence is known for LMW thiols and related molecules in this review.

***Francisella* and tularemia**

Francisella (F.) tularensis causes the disease tularemia, which is a zoonotic disease for humans (*1*), mainly occurring in lagomorphs and rodents in nature. It is also a Category A, Tier 1 select agent, due to its high infectivity via aerosol inhalation and its historical development as a biological weapon. It can be vector-borne or acquired via aerosol inhalation, consumption of contaminated water or by inoculating an open cut while handling an infected animal.

***Francisella* subspecies and intracellular lifestyle.**

The bacterium *F. tularensis* has 4 major subspecies (*1-3*). *F. tularensis* subspecies *tularensis* (Type A), the most virulent strain for humans, is a biosafety level 3 pathogen, and causes the majority of US cases of human tularemia (*1*). This includes the strain Schu S4 which is commonly studied. The subspecies *holartica* (Type B) is a less virulent form found commonly in northern Eurasian countries, such as Russia, Sweden, and Norway, as well as Japan, and causes most of the global cases of human tularemia (*1, 3-5*). A commonly used model strain of *F. holarctica* subtype is the *Francisella* Live Vaccine strain (LVS), which is not pathogenic for humans, but is highly homologous to the human pathogenic *F. holarctica* strains and to Type A *Francisella* (99.2%) and is lethal to mice (*4*). *F. tularensis* subspecies *mediasiatica* is a less common human and mammalian pathogen found in central Asia (*6, 7*). Lastly is the environmental subspecies *F. tularensis novicida* (also called *F. novicida*) whose genes are 97% homologous with the subsp. *tularensis* and causes disease in mice while being infectious only for immunocompromised humans (*7-9*). This strain is also commonly employed as a Biosafety Level 2 experimental model for the more virulent strains, along with the Live Vaccine strain (*3*). There are several other environmental species in the genus, including *F. philomiragia* and *F. noatunensis (10)*. *Francisella* species have been reported to infect over 250 species of animals, from birds to fish, cats, dogs, rodents, amoeba and humans (*5*), revealing the broad host range of this pathogen.

Francisella is a facultative intracellular Gram-negative bacteria which can achieve high levels of intracellular replication in the cytoplasm of phagocytic eukaryotic cells, such as macrophages (*1*) or non-phagocytic cells (*11*) during the course of the infection. *Francisella* can enter most eukaryotic cells, escape the phagosome via its Type 6 secretion system, and replicate to high numbers in the cytosol. No replication occurs in the phagosome.The only exception we are aware of is rat macrophages (*12*), which for unknown reasons do not support intracellular growth of *Francisella*. Although *Francisella* can be occasionally identified in the blood (albeit unreliably) during infection, it is predominantly found inside of eukaryotic cells while infecting a host, leading to cell death and pathogenesis (*13*). *Francisella* in the environment may not have this intracellular lifestyle, as it has been reported in water, mud, soil, rodent droppings and rodent carcasses (*1*), although it has been detected within single-cellular eukaryotic aquatic amoeba (*14-23*).

Francisella is auxotrophic for CSH and other amino acids.

Despite the wide range of organisms and environments in which this bacterium can be found, *Francisella* is surprisingly fickle with regards to *in vitro* laboratory growth (*2, 24, 25*). *Francisella* is auxotrophic for CSH and requires iron as well as supplementation of up to additional 12 amino acids including arginine, histidine, isoleucine, lysine, tyrosine, methionine, threonine and valine (*24, 26*). This bacterium requires a large exogenously supplied pool of these amino acids,especially CSH (CSH) for growth *in vitro* and for the robust intracellular proliferation that occurs in the cytosol of host cells (*24, 26*) where it must obtain these amino acids from the host during infection.

Nutritional Challenges of Intracellular growth for _Francisella._

F. tularensis is <u>*facultatively*</u> intracellular in eukaryotic cells, from amoeba to insect cells and from to mammalian macrophages to hepatocytes and epithelial cells (*19, 27*). Like *Rickettsia, Shigella* and *Listeria, Francisella* bacteria escape the phagosome to replicate to extremely high numbers in the host cytoplasm (*1, 28, 29*). This cytosolic intracellular niche provides the opportunity to acquire nutrients from the host cell's cytoplasm, to avoid the host immune system and antibiotics, and potentially to manipulate the host to better support bacterial growth (*13, 30, 31*). A complex array of interactions between the intracellular pathogen and the host determines the course of the infection, including induction of gene expression on both sides (*11*), release of protein effectors on the pathogen side, and activation of pathways such as autophagy and innate immunity for eradication of the bacteria on the host side (*1, 6*). For example, *Francisella* may induce an alternative (ATG5-independent) macro-autophagy within host cells to acquire additional amino acids to support its rapid rate of intracellular growth (*29*). For these intracellular bacteria, the host-cell cytosol may provide survival benefits, but it may also be nutritionally challenging, as the host may tightly sequester some much-needed nutrients or micronutrients (*30*), which is the basis of "nutritional immunity" (*31*). Recent studies in *Francisella* metabolism have begun to explore this important topic of nutritional interaction (*24, 26, 28, 29, 31, 32*), especially with regards to glutathione (GSH) (**Figure 1A-F).**

Glutathione is the major LMW thiol in _Francisella_

Glutathione (GSH) is a non-ribosomal thiol tripeptide, (γ-L-Glutamyl-n-cysteinylglycine) with an unusual γ-amide linkage that protects it from cleavage by most proteases except Gamma-Glutamyl Transpeptidase (Ggt). To assess which LMW thiols are produced in *Francisella*, we performed an assay to measure the presence of these thiols in two strains of *Francisella* grown in tryptic soy broth containing 0.1% CSHCSH (TSBC) for 18 hours (van Hoek and Rawat, *personal communication*). LMW thiols were measured by HPLC analysis of fluorescent thiol adducts with monobromobimane (*33*). The amount of thiol was normalized by dry weight of sample. The results (**Table 2**) identified GSH at approximately 3 µmol/g dry weight and CSHCSH at high levels as well. The levels of CSH in *Francisella* were high compared to other bacteria (*34*) likely due to the presence of required CSHCSH in the growth media, as CSH is usually undetectable in the cells since it is easily oxidized. Interestingly, *F. novicida* had almost 2.5-fold more CSH than *F. philomiragia* while the amount of GSH was very similar between the two species. The full chromatograms and data analysis are in the Supplemental information, **Figure S2** and **Table S1**.

Glutathione biosynthesis

The biosynthesis of GSH occurs through a two-step process which combines CSH, glycine, and glutamate to form the GSH tripeptide (*35, 36*). Like most gamma proteobacteria, *Francisella* has genes for both *gshA* and *gshB*. (**Table 1, Figure 1A-B**) (*2, 36-38*). The KEGG pathway and *F. tularensis* SchuS4 genes annotated to be part of GSH metabolism are shown in **Supplemental Figure S1** for the reader's interest. GshA, γ-glutamylCSH synthetase, ligates the amino group of CSH to the γ-carboxyl group of glutamate while GshB, GSH synthetase, condenses the resulting γ-glutamylCSH (γGC) with glycine to generate GSH (*39*). Both GshA and GshB proteins are in the *Francisella* cytoplasm (*2, 38*). In *F. tularensis* subsp. *holarctica* LVS, *gshA* and *gshB* are not essential *in vivo* in murine macrophage-like J774A.1 cells but are essential for *in vitro* growth on cystine heart agar supplemented with 1% hemoglobin(*38*), suggesting that the host cell can supply GSH. Although it is not proof of essentiality, no transposon insertion mutants for *gshA* and *gshB* were recovered in the *F. novicida* transposon library by Gallagher et al. (*40*). Similarly, *gshA* and *gshB* were found to be essential for *F. tularensis* Schu S4 growth *in vitro* (*41*).

Redoxin genes in *Francisella*

Other LMW thiols include glutaredoxins (Grxs) and thioredoxins (Trxs) (*42*), which are small heat-stable oxidoreductases that contain two conserved CSH residues in their active sites. They provide reducing equivalents for enzymes that form a disulfide as part of their catalytic cycle, such as ribonucleotide reductase (involved in DNA synthesis) *(43),* and protect against oxidative stress (*44, 45*) by providing reducing equivalents to peroxiredoxins like AhpC and thiol-peroxidase (Tpx) enzymes. Grx's and Trx's are structurally similar small proteins, have overlapping biochemical functions.

Glutaredoxins (Grxs)

Grxs are maintained in a reduced state by GSH, which is itself maintained in a reduced state by GSH reductase Gor (**Figure 1F**). Gor is present in *Francisella* cytoplasm but was not essential for virulence either *in vivo* or *in vitro* (**Table 1, Figure 1D**) (*2, 38*). There are 3 *Francisella* genes coding for glutaredoxins (**Figure 1F**) *(43, 46)* (**Table 1)**. In *Francisella*, expression of glutaredoxin 1 (*grxA*) gene is upregulated upon intramacrophage growth (*47*). GrxA enzyme is able to regenerate the reduced form of GSH following an oxidizing event (*35*). Glutaredoxin 2 (*grxB*) functions in metal binding and has oxidoreductase activity. *GrxC* is annotated as glutaredoxin 3. Ireland et al identified *grxA* and *grxC* genes as being essential for *F. tularensis* Schu S4 growth *in vitro* (*41*). Similarly, Asare *et al* showed a 2-log reduction for *grxA* mutants in human macrophage U937 cell line (*48, 49*) using the *F. novicida* transposon insertion mutant library (*40*). (*N.B.* The designation of *grxB* mutants as being defective in intracellular replication as well as FTN_0983 glutaredoxin 3/ ribonucleotide reductase beta-subunit was found to be an error in gene numbers (*49*).) On the other hand, in several other virulence screens in *Francisella*, no *grx* mutants were identified as having defects in intracellular replication or virulence (*50, 51*). Weiss *et al* made their own *F. novicida* transposon mutant library and screened it in mice following intraperitoneal infection and did not identify *grx* mutants (*50*). Similarly, using Gallagher *et al's F. novicida* transposon mutants in SualB cells (hemocyte-like cells from the mosquito *Anopheles gambiae*) no *grx* mutants were identified (*52*). Perhaps these differences reflect polar downstream effects of the mutations or reflect differences in the host

cell lines (insect versus mammalian). In summary, *grxA-C* were occasionally identified as being required for *Francisella* intracellular replication.

Thioredoxins (Trxs)

Bacterial thioredoxin and thioredoxin reductase, which maintain Trx's in a reduced condition, are important in the redox state in bacteria in addition to Grx's (*46, 53, 54*). These enzymes are encoded by the genes *trxA1*, *trxA2* and *trxB* respectively in *Francisella* **(Table 3)**, **(Figure 1E)** (*4*). Deletion of thioredoxin reductase gene *trxB (trxR)* showed very little impact in the *Francisella* oxidative stress response (*55*). A 2.5A crystal structure of the *Francisella* trxB protein has been deposited in the Protein Database (PBD 6BWT). Knockouts of the thioredoxin *trxA1* gene have shown significant impacts on the virulence of different pathogenic gram-negative bacteria (*44, 53, 56*). *trxA1* was identified as being critical in *Francisella* virulence studies (*57, 58*). Kraemer *et al* used *F. novicida* transposon mutants to screen for genes required for aerosol infection in mice and found that *trxA1* mutants showed attenuation during infection with a 2-log reduction in bacterial load at 24 and 48 hr time points compared with wild-type. Following this attenuation, the *trxA1* mutants began to be cleared from the lungs at 14 days post-infection. Interestingly, the initial concentration of bacteria in the aerosol directly correlated to the survival time for the mice (*57*). This suggests a dose-dependent lethality for *trxA1* in *F. novicida* in mice, which the researchers propose is a unique property compared to all the other genes screened (*57*). Despite their weakened ability to infect, *trxA1* mutants were still able to spread to the spleen and liver following inhalation (*57*). Another study reported a 3-fold reduction in growth inside U937 macrophages for *F. novicida trxA1* mutants (*49*). Together, these results indicate that thioredoxin gene *trxA1* is required for virulence in *F. novicida* and in intramacrophage infection. A recent study confirmed that in *F. tularensis LVS*, *trxA1* (FTL_0611) but not *trxA2* (FTL_1224) plays a major role in the oxidative stress response (*58*). A second thioredoxin gene *trxA2* is also present in the genome and may have a role in protecting against oxidative stress resistance over long periods of time (*58*). Interestingly, it has been proposed that the TrxR-Trx systems may be a target for antibiotic development in some bacteria (*54*).

***ahpC* protects against Oxidative stress and Nitrosative stress.**

Intracellular pathogens must be able to protect against the "respiratory burst" that releases reactive oxygen species (ROS), such as superoxides in the phagosomes of the macrophages (*59*) damaging DNA, lipids and proteins. The oxidative stress response in *Francisella*, like that in many other Gram-negative bacteria, uses mainly superoxide dismutases and catalases (*7, 36, 55, 58, 60-64*). The catalase-peroxidase *katG* (**Table 1**), which encodes a bifunctional enzyme with both catalase and broad-spectrum peroxidase activity is a known virulence factor of *Francisella* with mutants in *katG* being less pathogenic (*65*).

F. novicida also has two peroxidases, GSH peroxidase (Gpx) and FTN_1624 (a member of the DyP-type peroxidase family), and three putative peroxiredoxin enzyme genes: *ahpC* (FTN_0958) coding for an alkyl hydroperoxide reductase enzyme; AhpC/Prx1 sub-family (FTN_0973); and BCP/PrxQ sub-family (FTN_1756) (*66*). Of these, only *ahpC* peroxiredoxin gene is conserved among the human pathogenic strains of *Francisella* (**Table 3**) and this OxyR-regulated gene is protective against oxidative stress (*66*). The *ahpC* gene was also found to be essential for *F. tularensis* Schu S4's intracellular replication in bone-derived murine macrophages and Raw264.7 macrophages (but not the murine macrophage cell line J774) (*37,*

41, 66), while Ireland et al, 2019 found it to be an essential gene in *F. tularensis* Schu S4 (*41*). In addition, *ahpC* is required for virulence in mice in *Francisella* LVS infections (*66*), but is not essential in *F. novicida* for *in vitro* growth (*40*).

While most Gram-negative bacteria have an alkyl hydroperoxide reductase enzyme, AhpC, that requires AhpD or AhpF proteins as reductants to recycle the AhpC (*67*), *Francisella* does not contain *ahpD* and *ahpF*, and in this way is more similar to the Ahp system in the eukaryote *Plasmodium* which also lacks *ahpD* and *ahpF* (*66, 68*) (**Figure 1E**) and uses GSH as a reductant. *Francisella ahpC* is annotated as a GSH-dependent peroxiredoxin (**Table 3**), and may function similarly to the *Plasmodium* Aph system using GSH or perhaps Trx as reductants to recycle the AhpC (**Figure 1E**). The same *ahpC* gene is protective against nitrosative stress in *Francisella* LVS and Schu S4 as well as against oxidative stress since it can scavenge peroxynitrite as well as hydrogen peroxide as a substrate (*66*).

Glutathione protects against oxidative stress.

GSH can donate electrons to oxidants (*69*) or it can participate in electron relays involving a GSH peroxidase, peroxiredoxins, and thiol transferases where they donate electrons for the reduction of peroxides (*37, 70*). In the process, GSH is oxidized, where two GSH molecules form a disulfide bond to produce GSSG (oxidized GSH), which is reduced by Gor (**Figure 1D**) (*35*). In addition, GSH can protect exposed CSHs in proteins via *S*-glutathionylation (*71*). *S*-glutathionylation of an exposed CSH residue has been shown to be catalyzed by *Grx's* (*72*). The mixed disulfide bonds are reduced by Trxs and Grxs in a process called deglutathionylation (*42*) **(Figure 1F)** once the source of the oxidative stress is removed (*71*).

Glutathione and Nitrosative Stress

GSH also provides protection against nitrosative stress. Reactive nitric oxide levels can be modulated by GSH due to the formation of *S*-nitrosoglutathione (GSNO) and *S*-nitrosoglutathione reductases (GSNOR), which reduce the *S*-nitrosoglutathione to GSH and ammonia or other N species (**Figure 1C**). In *F. novicida*, the GSNOR *adhC* gene is FTN_0409. This protein was detected in *F. novicida* and *F. philomiragia* Outer Membrane Vesicles (*73*). However, this gene appears to only be present in the environmental species of *Francisella* (for example, in *F. philomiragia* as *Fphi_0427)* but is not present in the human pathogenic species such as Schu S4 or *F. holarctica* strains. Thus, this gene is likely not relevant to nitrosative stress response in human infection by tularemia.

Transcriptional regulation of oxidative stress response and GSH-pathway.

Oxidative stress response in *Francisella* is regulated via the global regulator MglA and OxyR (*7, 55, 58, 61, 64*). As in other Gram-negative bacteria, OxyR regulates the oxidative stress response in *Francisella* by regulating expression of over 125 genes (*55*). In *E. coli*, Grx and GSH can activate the OxyR oxidative stress regulator and begin the oxidative stress pathway (*53*). In *Francisella* OxyR protein regulates the expression of classical antioxidant enzyme genes *ahpC* and *katG* (*55, 65*). Proteins affected by an *oxyR* deletion in *F. tularensis* LVS include the oxidative stress resistance pathway such as AhpC (peroxiredoxin), KatG (catalase), SodB (FTL_1791), and TrxB (*55, 74*). These proteins all play critical roles in oxidative stress in *Francisella* LVS and Schu S4 strains (*37, 65, 66, 75*). Recently, it was found that the protein TrxA1 but not TrxA2 plays a major role in the oxidative stress response in *F. tularensis LVS*, such that *Francisella trxA1* regulates the expression of *oxyR*, subsequently regulating the

expression of the OxyR-dependent oxidative stress response genes (*58*). This contrasts with the *E. coli* model, in which OxyR enzyme is regulated by Grx and GSH (*76-78*). The transcriptional master-regulator of *F. tularensis*' virulence *mglA* (*79, 80*) controls the expression of many virulence genes in the *Francisella* pathogenicity island (*7, 16, 79, 81*). *mglA* also regulates the expression of many genes involved in responses to starvation and oxidative stress, including those for thioredoxin *trxA1*, glutathione synthetase *gshB*, two peroxiredoxins, *ahpC (FTN_0958)* and an "AhpC/TSA family member" (FTN_0973), *katG*, NADPH-quinone reductase (FTN_0840), and two glutaredoxins *grxB* and *grxA* (*7*).

In some of the environmental species of *Francisella* (those that are not human pathogens), additional genes involved in managing oxidative stress such as homologues of the MarR family organic hydroperoxide resistance transcriptional regulator, *ohrR*, can be identified (e.g., Fphi_1391), but these genes are not conserved in the human-pathogenic strains and so will not be discussed further.

Thus, there are at least two transcriptional regulators identified in *Francisella* which control the genes involved in oxidative stress response (*mglA* and *oxyR*). Whether these regulators can be directly or indirectly affected by GSH in *Francisella*, similar to that shown in *B. pseduomallei* (*82*) and *L. monocytogenes* (*83*) remains to be determined, with the regulation of *Francisella oxyR* by *trxA1* being an important advance in our understanding.

Gluthathione from host cells as a source of CSH for *Francisella*

GSH from host cells is an important reservoir of CSH and thus may be an important host source of nutrition for *Francisella* (*2, 38*) (**Figure 1A**). GSH is at high levels in eukaryotic cell cytosols (estimated between 0.5-15 mM)(*84*) and could be transported into the *Francisella* periplasm via porins such as FupA (*85*), potentially providing *Francisella* the chance to acquire much needed CSH for which it is auxotrophic (*24*). (**Table 1**). The gamma (γ) linkage between the CSH and glutamate residues is uniquely cleaved by Ggt enzyme (**Table 1, Figure 1A**) to yield glutamate and Cys-Gly (L-cysteinyl-glycine) (*2, 35*). Cys-Gly is further processed to CSH and glycine by other peptidases (*2, 86*). The subcellular localization of *Francisella* Ggt enzyme is within the periplasm (membrane anchored) (*38*), which would facilitate its proposed activity against host derived GSH (**Figure 1A**).

Alkhuder *et al*, 2009 first provided evidence that the *ggt* gene is required for intracellular cytosolic replication of *Francisella* and suggested that *F. novicida* utilizes GSH from the host cell cytosol to recover CSH, thus "glutathione stealing" (*2, 40, 51*). The studies demonstrated the impaired replication of the *Francisella* LVS *ggt* mutant (FTL_0766) in J774, RAW, and bone-derived macrophage cells (*2*), and was confirmed by other labs (*87*). Other studies confirmed a role for *ggt* in *F. tularensis* Schu S4 replication in HepG2 cells (*88*). Furthermore, *ggt* mutants are less virulent in BALB/C mice (intraperitoneal route of infection) by three orders of magnitude (*2*). Alkhuder et al, 2009 also demonstrated that the CSH from GSH was incorporated into *Francisella* proteins demonstrating that GSH can be used for catabolism and relieve the CSH auxotrophy (*2*). Contrasting reports find that the *ggt* gene is not essential for *in vitro* growth of *Francisella* LVS on cystine heart agar supplemented with 1% hemoglobin (*38*), but these screens were done on CSH and iron rich media, so the phenotype may not have been apparent. Ireland *et al* also found that *ggt* was essential for *F. tularensis* Schu S4 growth *in vivo* in the Fisher 344 rat spleen (*41*). Interestingly, the optimal catalytic temperature for bacterial Ggt enzyme was determined to be between 37-60°C (*89*). A temperature of 37°C would reflect the

localization *Francisella* bacteria inside a mammalian host versus an insect vector (22°C) or an environmental location (<22°C).

Cytosolic aminopeptidases PepN, PepB, and/or PepA can process Cys-Gly into glycine and CSH respectively (**Figure 1A)** in a similar manner as proven in *E. coli* (*2, 86*). These metabolic products can then be recycled back into GSH or be used for other nutritional functions. The actions of Ggt and aminopeptidases together would enable *Francisella* to utilize GSH if CSH or glutamate are low, as suggested by Alkhulder et al, 2009 (*2*).

DptA, the Cys-Gly transporter.

DptA, the Cys-Gly dipeptide transporter, was identified in *F. tularensis* LVS (FTL_1251, FTT_0953c, FTN_0832) (*38*) (**Figure 1A**), exhibiting partial growth defects when grown with Cys-Gly as the only sulfur source. DptA (Dipeptide and tripeptide permease A), is an inner membrane proton-dependent oligopeptide transporter-family (POT) symporter protein of the Major facilitator (MFS) superfamily. As shown in **Table 3**, *dptA* is required for *intracellular* replication in *Francisella* LVS (*38*) and infection in *F. tularensis* Schu S4 (*41*)The peptide transporter *dptA* is not essential in LVS during *in vitro* growth but is required for intramacrophage growth (*38, 41*) and mutants strongly affect *F. tularensis* SchuS4 and replication in macrophages, epithelial cells and in the Fisher rat infection model (*41*). When Holland et al compared *F. tularensis* LVS in BHI media pH6.8 (representing host-adapted, infection conditions) versus MHB media (representing nonhost-adapted conditions), *dptA* demonstrated an induction ratio of f 202.2 fold, the second most highly changed gene after uridylate kinase in their study(*90*). Gene expression of *dptA* was increased during infection (*47, 91*). In contract, inactivation of *dptA* did not exert a high fitness cost for *F. novicida,* (*85*).

ChaC, a GGCT (γ–glutamyl-cyclotransferase) enzyme involved in degradation of GSH.

A new Tn-Seq study in *Francisella* LVS was published by Ramsey *et al* (*38*), which highlighted the central role of GSH metabolism in *Francisella* virulence and intracellular replication and identified new genes and proteins involved in this pathway. The recently characterized periplasmic (membrane anchored) protein in *F. novicida* and *Francisella* LVS is ChaC, an enzyme that is capable of cleaving GSH to 5-oxoproline and the dipeptide Cys-Gly, in contrast to the Ggt reaction (*38*) (**Figure 1A**). Ramsey *et al* demonstrated that the *chaC* gene is required for intracellular growth of *Francisella* in the murine macrophage-like J774A.1 cell line (*38*). This study determined that *ggt and chaC,* and the aminopeptidase *pepA* genes were all found to be required for *in vivo* replication for *Francisella* LVS (*38*). In the Fisher 344 rat model, *ggt, pepA,* and *chaC* were also found to be required for infection by *F. tularensis* SchuS4 (*41*) (Table 3).

FupA, an import porin, and NgtA, a Major facilitator superfamily (MFS) glutathione transporter in *F. novicida.*

Recent studies by Wang et al (*85*) have identified the outer membrane porin FupA (FTN_0444) as the import porin for intact GSH or Cys-Gly dipeptide for *Francisella*. This could provide a mechanism of GSH import (aka "stealing") from host cytosol or other GSH-rich environments. FupA is present on Schu S4 and LVS as well (Table 3). Transposon insertion mutants in FTN_0444 were four logs less capable of intracellular replication in U937 human macrophages (*49*). Wang et al (*85*) also identified another GSH transporter of the MFS superfamily, *ngtA* (FTN_1011). NgtA is a 12 transmembrane protein found on the inner

membrane exclusively in intracellular pathogens. There are homologous genes in the other strains of *Francisella*, see **Table 3**. In the absence of GSH cleavage in the periplasm, this transporter appears to be able to transport the intact GSH tripeptide to the cytoplasm, highlighting a role of GSH uptake for environmental species of *Francisella*.

Wang et al (*85*) also newly identified a cytoplasmic glutamine amindotransferase (GATase) enzyme, *cgc1/cgaA* (FTN_0435), that is capable of GSH breakdown in *F. novicida*. The presence of this pathway may provide parallel pathways for GSH catabolism in *F. novicida* (unlike *F. tularensis* Schu S4 and LVS) during intracellular infection of macrophages to maintain GSH homeostasis. Transposon insertion mutants in FTN_0435 were four logs less capable of intracellular replication in Drosphila S2 cells (*49*). The presence of this parallel pathway in the one strain and not the others may also explain some of the differences observed in animal infection studies for *ggt* mutants in *F. novicida* vs *F. tularensis* Schu S4 and LVS, and perhaps relates to the differences in human pathogenesis between the different strains.

***Francisella*'s Use of GSH to sense Intracellular/host localization.**

The ability to transport ("glutathione stealing") and use GSH is essential for intracellular cytosolic proliferation of *Francisella,* despite its ability to synthesize GSH. Potential transporters, DptA and GadC proteins (glutamate importer) (**Figure 1A**), are present on *Francisella*'s cytoplasmic membrane (*36, 38*) to aid in "glutathione stealing". Both transporters move their cargo (the dipeptide or glutamate) from the bacterial periplasm to its cytoplasm (*38*). In the presence of high levels of exogenous GSH (glutathione) such as when *Francisella* is in the cytosol of a macrophage, the excess tripeptide GSH would be cleaved by Ggt and/or ChaC enzymes in the periplasm to the di-peptide L-cysteinyl- glycine, which is then transported inside *Francisella* via DptA to provide CSH and glycine for catabolism and growth. Alkhulder et al, (*2*) and subsequently Ramsey *et al* (*38*) demonstrated that the CSH from host GSH ends up in bacterial proteins, proving that this "glutathione stealing" pathway occurs. One speculation is that the bacterium could potentially "sense" its intracellular/cyotosolic host location by the high periplasmic levels of glutamate resulting from Ggt cleavage of host-derived GSH, which are then transported to the cytoplasm via GadC, leading to high intracellular levels of glutamate (*36*) and the increased intracellular production of the low MW thiol GSH. This process could be further promoted by the maximal catalysis of Ggt at the host body temperature of 37ºC. The recently demonstrated GSH-binding NgtA protein (*85*) could also provide a direct mechanism for the periplasmic GSH to be transported to the cytosol and detected by *F. novicida* (Table 3). Alternatively, the bacteria could sense the high levels of di-peptide. The GSH-cleaving genes *ggt* and *chaC* genes are not essential for *F. tularensis* Schu S4 *in vitro* growth in bacterial media (*38*). Deletion of *chaC* had no reported effect in the Fisher rat *in vivo* infection model; however, *ggt* mutants make the bacteria strongly attenuated *in vivo*, for replication in rats, mice, macrophages and hepatocytes (*41*). Notably, transposon mutants in *F. novicida gadC* showed a 4-5 log reduction in replication in U937 cells (*49*) and *gadC* is also required for *in vitro* growth of *Francisella LVS* (*36, 38*).

***In vitro* Growth and self-production of GSH**

Since *Francisella* is a *facultative* intracellular pathogen, not *obligate*, it does have the ability to grow independent of the eukaryotic host. Low levels of Ggt are produced in the cytoplasm of bacteria grown in their optimal broth, unless they are grown under stress conditions, such as nutrient limitation (*85*). Bacterially produced GSH is proposed in this model

to be exported to the periplasm via CydD, the proposed tripeptide exporter (*38*) (**Figure 1A**). In *E. coli*, CSH-GSH ABC transporter CydD is a transporter which can export GSH from the cytoplasm to the periplasm (*92*). In virulent *Francisella*, *cydD* is required for *in vitro* growth, but not *in vivo* growth (below). *Francisella*, like all bacteria, needs GSH in the periplasm for the redox balance that would allow folding of such proteins as cytochromes b/d. For *in vitro* growth, GSH would have to be exported out to the periplasm once it is made in the cytoplasm. *In vivo*, GSH could be transported into the periplasm from the host via an outer membrane porin such as FupA (*85*) and thus *cydD* would not be required. The *cydD* gene is essential for *in vitro* growth in *F. tularensis* Schu S4 and LVS, not essential for Schu S4 or LVS intramacrophage replication and not essential for Schu S4 *in vivo* growth as its deletion had no effect in the Fisher rat infection model (*41*)(Table 3). Combined, these points suggest that the **autologous production and export** of GSH are critical to virulent *Francisella*'s *in vitro* growth in CSH-supplemented media while *in vivo* or intracellular replication requires the **import** of the di-peptide product of GSH degradation, potentially from **exogenous/host GSH**. Confusingly, *cydD* is not required for *in vitro* growth in the relatively avirulent strain *F. novicida* (*40*). These differences still require some additional study to understand if there are different pathways present in the different strains, such as suggested by the recent discovery of the role of *fupA/NgtA/cgaA* in *F. novicida (85)*, and if these differences relate to the differences in virulence towards humans.

Interestingly, as mentioned previously, the glutamate transporter GadC is also required for *in vitro* growth of *Francisella LVS* (*36, 38*), perhaps to balance the exogenous CSH from the media and to make GSH. Additional genes in this pathway which are required for *Francisella* LVS *in vitro* growth include *gshA, gshB and cydD*. The genes mentioned in the prior section (*dptA*, *chaC* and *ggt*) are all also required for intracellular replication of *Francisella* and infection of rats with Schu S4 (*36, 38, 41*). Thus, *Francisella* may sense the amount of glutamate or GSH in its environment as a signal of its intracellular localization via the GadC transporter. **Limited periplasmic glutamate levels could be a signal that it is in an environmental location such as water or mud**. *Francisella* could then transport any available environmental glutamate to the inside of the cell to enable cytoplasmic GSH biosynthesis (*26, 36*). This finding suggests that under environmental or *in vitro* growth conditions, *Francisella* would predominantly produce its own GSH from imported glutamate, since exogenous GSH levels would be low. This also highlights the importance of glutamate to support *Francisella* replication outside the host (*93*).

Francisella may be able to use the high levels of GSH or its degradation products (glutamate and the L-cysteinyl-glycine dipeptide) to detect if it is in the intracellular cytosol of the host cell. Conversely, the bacterium may be able to detect if it is in a free-living, environmental location by low levels of GSH, glutamate and/or Cys-Gly dipeptide.

Francisella LMW thiol related genes and essentiality studies

A thorough review of the *Francisella* literature surveying all the proteomic and transcriptional literature on each *Francisella* gene to the date of publication was done by Holland *et al* in a very substantial table in the supplemental material of their recent article (*90*). We have summarized that dataset for the genes/proteins relevant to GSH in **Table 3** with some updates. Additionally, data from transposon sequencing (Tn-seq) and RNA-seq data were compiled from screens for virulence in macrophage infection, essential *in vitro* genes, and reactions to the stringent response and added to Table 3. In this compilation table, expression studies or mutants of GSH synthesis and metabolism genes, thioredoxin pathway genes, and glutaredoxin systems

show notable results for Schu S4, LVS, and U112 strains of *Francisella*, most of which have been described in the relevant sections above. For example, the proteomics study by Holland *et al* demonstrated an almost 2-fold increase in TrxA1 protein expression and a 3-fold increase in TrxA2 protein levels when *Francisella* is grown in Brain Heart Infusion broth, pH 6.8 to mimic intra-macrophage conditions (*90*). Also of interest, the study by Murch *et al* highlighted the downregulation of several GSH-related genes under stringent response including *gshB, GSHP, grxA, grxB, pepB* and *trxB* (*94*).

In a different approach, using systems biology modeling to identify genes important in *Francisella* host-pathogen interactions, neither the GSH/Ggt pathway nor the thioredoxin pathway were predicted to be required for intracellular growth (*95*) in contrast to the experimental results found when testing relevant mutants (*2*). The systems biology modeling may inadequately "understand" the metabolic pathways in *Francisella* and having additional gene expression data could clarify the *in silico* model of metabolism during intracellular growth.

Conclusions:

Low molecular weight (LMW) thiols play a critical role in preserving a reducing environment within cells and facilitating electron transfer to diverse enzymatic systems. They are also essential in safeguarding cells against reactive oxygen and reactive nitrogen stresses. We reviewed the interconnected roles of GSH synthesis, detection, and transport in *Francisella* species and their potential role in overcoming the nutritional challenges of intracellular and planktonic growth.

The capacity of intracellular bacteria to discern their intracellular localization is fundamental to their ability to modulate gene expression and metabolism, thereby adopting a host-adapted phenotype. GSH, the primary antioxidant molecule generated by host cells, is utilized by various bacteria for protection against host reactive oxygen species (ROS) attacks and adaptation to the host environment. *Francisella* may employ GSH or Ggt degradation products (glutamate and the Cys-Gly dipeptide) to determine whether it is in an intracellular location (within a host cell) based on the presence of high GSH concentrations. Conversely, the bacterium may be able to detect if it is in a free-living, environmental location by low levels of GSH, glutamate and/or dipeptide.

GSH stealing and sensing is an attribute of other intracellular bacteria also. In *Listeria monocytogenes* allosteric binding of GSH to PrfA (*96*) leads to the expression of virulence determinants. *Burkholderia pseudomallei*, uses its VirAG two-component system to sense GSH and induce type VI secretion system expression (*82*). Thus, the molecular mechanisms underlying the sensing appears to be unique to each of these organisms. Other pathogenic bacteria which lack low molecular weight thiols such as GSH, mycothiol, or bacillithiol, use host-derived GSH to protect against oxidative stress, and thus have mechanisms for GSH import. In *Streptococcus pyogenes*, which is unable to synthesize GSH, GSH transporter GshT is essential for aerobic growth and inactivation of *gshT* leads to oxidative stress (*97*). *Hemophilus influenzae* imports GSH using an ATP-binding cassette (ABC)-like dipeptide transporter and a periplasmic-binding protein and lack of GSH in the growth media leads to oxidative stress (*98, 99*). The mechanisms for importing GSH vaybetween microorganisms.

While *Francisella* can synthesize GSH itself, it has many mechanisms to obtain it from exogenous sources which it appears to be prefer to *de novo* synthesis when the organism is *in vivo*. Recent studies have identified the outer membrane porin FupA as the import porin for intact GSH or Cys-Gly dipeptide and identified in *F. novicida* that NgtA protein plays a role for

importing these peptides to the cytoplasm from the periplasm. Additionally, periplasmic GSH-cleaving enzymes, Ggt or ChaC, which are required for *in vivo* replication, along with the dipeptide transporter DptA, can cleave GSH and deliver the "stolen" Cys-Gly peptide to the *Francisella* cytoplasm. GadC, the glutamate importer, is required for *in vitro* replication and is important in GSH metabolism in *Francisella*. Conversely. the tripeptide GSH can be exported from the cytoplasm via CydC/D proteins to modulate intracellular levels. The ability of *Francisella* to use GSH and its degradation products in these ways likely contributes to its success as a facultative intracellular pathogen. A deeper understanding of GSH sensing and metabolism in *Francisella*'s lifecycle is expected to provide insights into host-pathogen interactions and may reveal new targets for therapeutic intervention for tularemia infections.

Unanswered Questions:
Some additional questions that may be of interest for future research in this area include:
i. What are the potential functions of the multiple glutaredoxins in *Francisella*?
ii. There is a clear difference in the roles of *trxA1* versus *trxA2*. Further elucidation of the mechanistic role of each of these thioredoxins would be helpful. Unlike in *E. coli*, *Francisella trxA1* regulates the expression of *oxyR*, which then regulates the expression of the OxyR-dependent oxidative stress response (*55, 58, 74*). It is not known whether *Francisella* OxyR protein is itself a redox-sensing regulator, and whether it may be dependent on glutaredoxin to reduce the disulfide bonds in *Francisella* OxyR as part of OxyR-deactivation.
iii. The OxyR-regulated, GSH-dependent peroxiredoxin gene *ahpC* is protective against oxidative and nitrosative stress in *Francisella sp.* (*66*). Further work elucidating the reductants that recycle the *Francisella* AhpC protein is needed. Since *ahpD* or *ahpF* are not present in *Francisella sp.*, it would be useful to know whether *Francisella* AhpC relies on GSH or Trx as reductants to recycle its AhpC (*66*).

Acknowledgement: We are grateful to Jason Thomas, Fresno State University, for performing the thiol assay of the *Francisella* samples.

Figure and Tables:

Figure 1: Low MW Thiols in *Francisella*. Host-derived or exogenous GSH can be transported into the periplasm (**Figure 1A**), potentially by FupA GSH porin in the OM (not shown). From within the periplasm, in the presence of high levels of exogenous GSH, such as in the cytosol of a macrophage or from a host, the tripeptide GSH could be cleaved by Ggt and/or ChaC enzyme to the di-peptide L-cysteinyl- glycine, which is then transported inside *Francisella* cytoplasm via DptA to provide CSH and glycine for catabolism and growth. ChaC cleaves GSH to Cys-Gly and 5-oxoproline (not shown). Potential transporters, DptA (a Cys-Gly dipeptide transporter) and GadC (a glutamate transporter) (**Figure 1A**), are present on *Francisella*'s cytoplasmic membrane.

The synthesis of GSH is through a two-step process which combines CSH, glycine, and glutamate to form the tripeptide. The *Francisella* glutamate-CSH ligase protein, GshA catalyzes the first step in the two-step pathway, the reaction of L-CSH with L-glutamate to generate L-γ-glutamyl-L-CSH (**Figure 1B).** GshB (GSH synthetase enzyme) catalyzes the subsequent reaction of glycine and L-γ-glutamyl-L-CSH to generate GSH. GshB is in the bacterial cytoplasm, along with GshA (glutamate-CSH ligase). Bacterially produced GSH can be exported to the periplasm via CydD protein, the proposed tripeptide exporter (**Figure 1A**) to maintain homeostasis.

Reactive nitric oxide levels can be modulated by GSH due to the formation of *S*-nitrosoglutathione (GSNO) and the action of *S*-nitrosoglutathione reductases (GSNOR) (**Figure 1C**) which reduce the *S*-nitrosoglutathione to GSH and ammonia or other nitrogen species.

The reactive thiol group of GSH allows it to provide protection against oxidative stress, in which two GSH molecules provide reducing equivalents to the oxidants and are oxidized to GSSG in the process (**Figure 1D, F**). The enzyme, glutathione reductase (Gor) uses reducing equivalents from NADPH to reduced GSSG to GSH, restoring its function. The other component of the GSH system consists of glutaredoxins (Grx), which are small oxidoreductases, typically possessing two active CSH residues in the active site (**Figure 1D, F**). These Grx enzymes operate in tandem with GSH and NADPH, supporting an electron relay from NADPH through GSH to Grx to provide a flow of electrons to enzymes such as ribonucleotide reductase, which is involved in DNA synthesis in *E. coli*. S-glutathiolation of an exposed CSH residue is catalyzed by Grx. Thus, GSH prevents permanent protein damage by reactive oxygen species.

Thioredoxins have been shown to catalyze the denitrosylation of S-nitrosoglutathione and protein S-nitrosothiols to their reduced state, generating HNO as a by-product. Thioredoxin reductase maintains thioredoxin in a reduced state by facilitating the transfer of electrons from NADPH to the oxidized protein. (**Figure 1E**).

Symbols: ** essential in vivo, # essential in vitro,

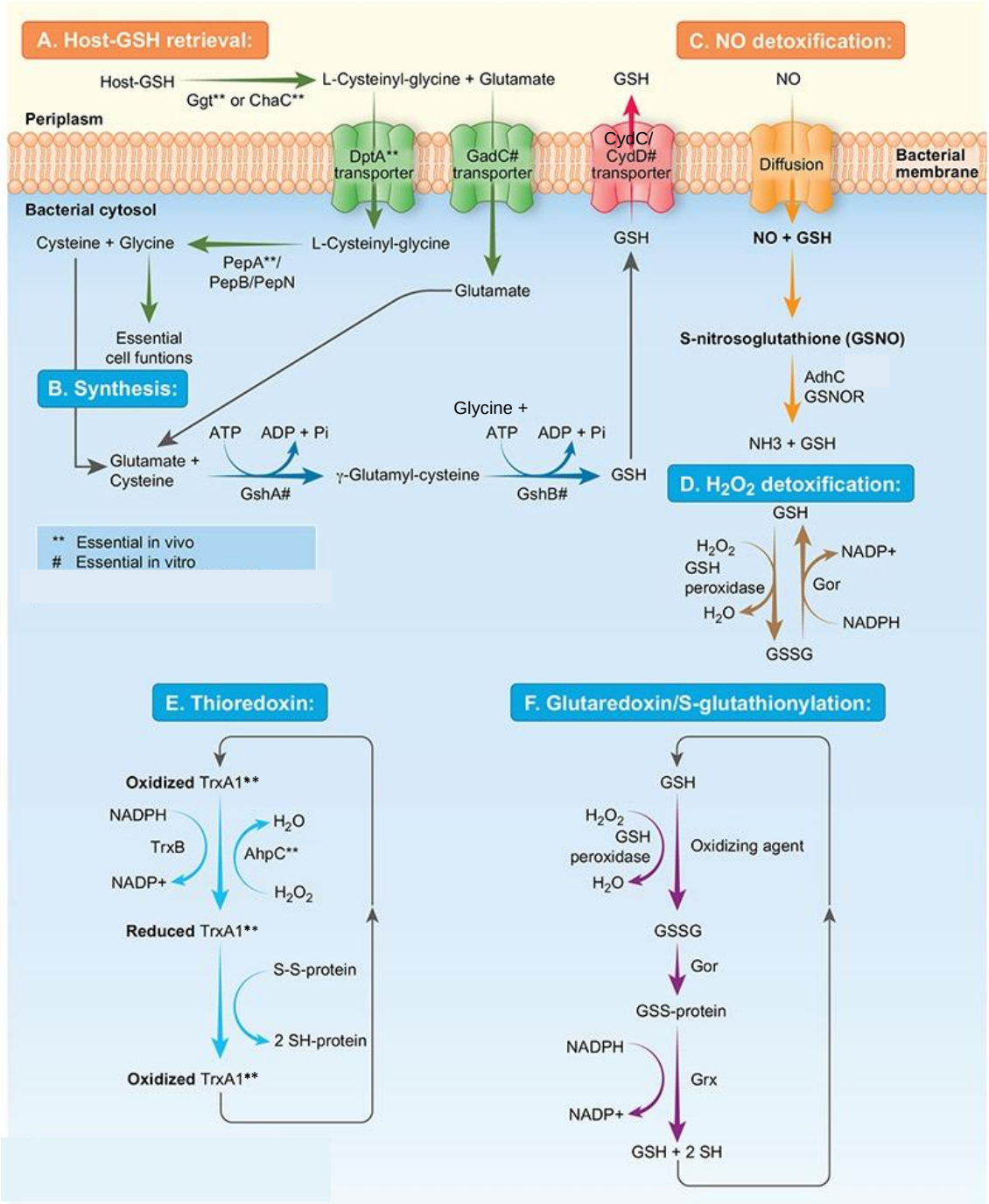


Table 1: Glutathione/GSH metabolism genes in *Francisella*. ** Essential *in vivo*, # Essential *in vitro.* E.C.= Enzyme Commission number; K=KEGG numbers.

Gene	*Francisella tularensis* subsp. *tularensis* (strain Schu S4)	*Francisella tularensis* subsp. *novicida* (strain U112)	*Francisella tularensis* subsp. *holarctica* (strain LVS)
Intra-bacterial Glutathione/GSH biosynthesis (cytoplasmic) (Figure 1A)			
Glutamate-CSH ligase, *gshA* # [EC:6.3.2.2] (K01919)	FTT_0367c	FTN_0277	FTL_1304
Glutathione synthetase, *gshB* # [EC:6.3.2.3] (K01920)	FTT_0926	FTN_0804	FTL_1284
Periplasmic GSH Cleavage to L-cysteinyl glycine (di-peptide) (Figure 1A)			
γ-Glutamyl transpeptidase, *ggt* ** [EC:2.3.2.2, 3.4.19.13] (K00681)	FTT_1181c	FTN_1159	FTL_0766
γ–glutamyl-cyclotransferase (GGCT), *chaC* ** [EC: 4.3.2.9]	FTT_0509c	FTN_0599	FTL_1548
Di-peptide transport from Periplasm to Cytosol (Figure 1A)			
dptA ** amino-acid/di-peptide transporter family protein; (K03305) (di-or tripeptide:H+ symporter)	FTT_0953c	FTN_0832	FTL_1251
gadC # glutamate:γ-aminobutyrate (GABA) exchanger (K20265)	FTT_0480c	FTN_0571	FTL_1583
GSH Export from Cytosol to Periplasm (Figure 1A)			
CSH/GSH ABC transporter permease/ATP-binding protein *cydD#* (K16013)/*cydC* (K16012)	FTT_1335/FTT_1336#	FTN_0642/ FTN_0641	FTL_1495/FTL_1496#
Cytosolic L-cysteinyl glycine (di-peptide) Breakdown (Figure 1A/B)			
cytosol leucyl Aminopeptidase A, *pepA*** [EC:3.4.11.1] (K01255)	FTT_1318c	FTN_0660	FTL_1479
cytosol leucyl Aminopeptidase B, *pepB* [EC:3.4.11.1] (K01255)	FTT_1094c	FTN_0780	FTL_1108 (DA46-1685)
membrane alanyl Aminopeptidase N, *pepN;* [EC:3.4.11.2] (K01256)	FTT_1793c	FTN_1768	FTL_1956
Intra-bacterial Glutathione/GSH biosynthesis (cytoplasmic) (Figure 1B)			
Glutamate-CSH ligase, *gshA* # [EC:6.3.2.2] (K01919)	FTT_0367c	FTN_0277	FTL_1304
Glutathione synthetase, *gshB* # [EC:6.3.2.3] (K01920)	FTT_0926	FTN_0804	FTL_1284
NO detoxification (Figure 1C)			
adhC	-	FTN_0409	-
H_2O_2 detoxification (Figure 1D)			
Glutathione reductase, *gor* [EC: 1.8.1.7] (K00383)	FTT_0955c	FTN_0834	FTL_1248
Glutathione peroxidase [EC:1.11.1.9] (K00432)	FTT_0733	FTN_0698	FTL_1383
Redoxin genes in *Francisella* (Protein disulfide isomerases).			
Thioredoxins (Figure 1E)			
trxA1#; thioredoxin (K03671)	FTT_1445	FTN_1415	FTL_1224
trxA2; thioredoxin (K03671)	FTT_0976	FTN_0856	FTL_0611
trxB/trxR; thioredoxin reductase (K00384)	FTT_0489c	FTN_0580	FTL_1571
Glutaredoxins (Figure 1F)			
grxA#; glutaredoxin 1 (K03674)	FTT_0533c	FTN_0982	FTL_0985
grxB; glutaredoxin 2 (K03675)	FTT_0650c	FTN_1033	FTL_0923
glutaredoxin-like protein *grxC#*; glutaredoxin 3 (K03676), [EC:1.17.4.1]	No homolog	FTN_0762	No homolog
Ribonucleoside-diphosphate reductase beta chain [EC:1.17.4.1] / glutaredoxin 3 (K00526/ K03676) #	FTT_0532c	FTN_0983 (*49*)	FTL_0984
grxD; monothiol glutaredoxin (K07390)	FTT_0067c	FTN_1643	FTL_1792
dsbB, disulfide bond formation protein [EC:1.8.5.9] (K03611) (not mentioned in Figure)	FTT_0107c	FTN_1608	FTL_1670
Peroxidase:			
Catalase/peroxidase, *katG;* [EC:1.11.1.21] (K03782)	FTT_0721c	FTN_0633	FTL_1504

Table 2: High levels of GSH are found in *Francisella novicida* and *Francisella philomiragia* 25017.

	CSH	GSH
Sample	**µmol/g dry weight**	
Francisella novicida	7.89 ± 0.51	3.00 ± 0.20
Francisella philomiragia 25017	3.19 ± 0.12	3.05 ± 0.09

Table 3: Summary of proteomic, Tn-seq, and RNA-seq data regarding *Francisella* GSH-related genes of interest for 3 strains. Adapted from Holland *et al (90)*. Symbols: ** essential in vivo, # essential in vitro, $ essential in Macrophage infection.

Type of Data			**Proteomics**	**RNA-Seq**	**Transcriptomics**	**Tn-Seq**		**TraDIS**
Source & Strain	**Francisella LVS and Schu S4 (*65*).**	***F. novicida* (*40*); Schu S4 (*100*); LVS (*101*).**	**Holland et. al (LVS and Schu S4) (*90*).**	**Murch et. al (Schu S4) (*94*).**	**Bent et. al (LVS) (*91*).**	**Ramsey et. al (*38*) (LVS unless otherwise indicated).**		**Ireland *et al* (Schu S4) (*41*).**
Gene ID	**Gene Function**	**Locus number (*novicida*); (SchuS4); (LVS)**	**Normalized Fold Change BHI/MHB**	**Change Under Stringent Response**	**4hr/8hr infection**	**Essential in Macrophage Infection**	**Essential for *in vitro* growth**	**Essential *in vivo* (rat)**
oxyR	Regulator of Oxidative Stress Response	FTN_0959; FTT_0556; FTL_1014.	1.5	No data	No Change at 4 hr/ Up Regulated at 8 hr	Not essential (*38*)	Not essential	No effect reported (*41*)
mglA #/$	Master Regulator of FPI Genes	FTN_1290; FTT_1275; FTL_1185.	0.6	No data	No Change at 4/8 hr	Not essential (*38*) Essential in Fn replication in U937 cells (*49*)	Not essential LVS (*38, 102*) Essential SchuS 4 (*41*)	No effect reported (*41*)
gshA #	Catalyzes first step of GSH synthesis	FTN_0277 (No mutants recovered); FTT_0367c; FTL_1304.	2.0	No data	No Change at 4/8 hr	Not essential (*38*)	Not essential (*38, 102*) Essential Schu S4 (*38, 41*)	No effect reported (*41*)
gshB #	Catalyzes second step of GSH synthesis	FTN_0804 (No mutants recovered); FTT_0926; FTL_1284.	1.7	No data	Down regulated at 4 hr/ No Change at 8 hr	Not essential LVS (*38*)	Not essential LVS (*38, 102*) Essential Schu S4 (*38, 41*)	No effect reported (*41*)
gshpx/ gpx	Glutathione peroxidase	FTN_0698 Not essential; FTT_0733; FTL_1284.	0.8	No data	No Change at 4/8 hr	Not essential (*38*)	Not essential (*38*)	No effect reported (*41*)
Peroxidase	Dyp-type peroxidase family protein	FTN_1624; FTT_0086; FTL_1773.				Not essential (*38*), Essential in Fn replication in U937 and S2 cells (*49*)	Not essential.	No effect reported (*41*)
adhC	S-Nitroso glutathione reductase	FTN_0409 Not essential; Not found in Schu S4 strain, present as BZ14_1978; Not found in LVS, fragment as AW21_1239.	Not present in LVS	Not present	No data	No data (*41*)	Not found	Not present
*ahpC $/**/#*	Glutathione-dependent peroxiredoxin	FTN_0958 Not essential; FTT_0557; FTL_1015.	1.7	No data	Down regulated at 4 hr/ No Change at 8 hr.	Not essential LVS in Mø (*38, 66*), Essential SchuS4 (*37, 41, 66*), Essential in Fn replication in U937 and S2 cells (*49*)	Not essential LVS (*38*) Essential Schu S4 (*41*)	Essential murine LVS infection (*66*) No effect reported for Schu S4 in rat (*41*)

ahpC/ TSA		FTN_0973 (*103*) Not essential; Not found in Schu S4 or LVS.	Not present	Not present	No data	Not present	Not present	Not present
Gor	Glutathione reductase	FTN_0835 Not essential; FTT_0955c; FTL_1248.	1.1	No data	Down regulated at 4 hr/ No Change at 8 hr	Not essential (*38*)	Not essential (*38*)	No effect reported (*41*)
*ggt***/$*	Gamma-glutamyl trans-peptidase	FTN_1159 Not essential; FTT_1181c; FTL_0766 * Intracellular growth defect.	0.9	No data	Up Regulated at 4 hr / No Change at 8 hr	Essential (*2, 38*) (-4.7 log2FC)	Not essential (*38*)	Essential for rat infection (*41*). Also macrophages, mouse, hepatocytes.
cydD/ cydC	CSH/ GSH ABC transporter permease/ATP-binding protein	FTN_0642/ FTN_0641; FTT_1335/FTT_1336; FTL_1495/FTL_1496.	1.6	No data	Down regulated at 4 h (0.38) and upregulated at 8 hr (1.35)	Not essential (*38*)	Not essential *F. novicida* (*40*), & Schu S4 (*41*) *in vitro*	No effect reported (*41*)
chaC $	γ–glutamyl-cyclotransferase (GGCT) enzyme	FTN_0599; FTT_0509c; FTL_1548.	0.6	No data	No Change at 4/8 hr.	Essential (*38*) (-8.8 log2FC)	Not essential Schu S4	No effect reported (*41*)
dptA **/$	amino-acid/di-peptide transporter family protein (K03305) (di-or tripeptide:H+ symporter) POT (Proton-dependent oligopeptide transport family protein)	FTN_0832 FTT_0953c FTL_1251	**202.2**	No data	Up Regulated at 4 hr (3.81) and at 8 hr (2.41). Also upregulated at 2, 4, 8, 12, 16, 24 hrs (*47*)	Essential (*38*) (-4.94 log2FC)	Not essential LVS (*38*)	Essential for rat infection (*41*). Also macrophages, and Epithelial cells.
gadC #/$	glutamate: γ-aminobutyrate (GABA) exchanger	FTN_0571 FTT_0480c FTL_1583	No data	No data	Up Regulated at 4 hr (1.6) but Down at 8 hr (0.73).	Not essential (*38*), Essential in Fn replication in U937 and S2 cells (*49*)	Essential in LVS (*38*)	No effect reported (*41*)
grxA #/$	Glutaredoxin A	FTN_0982; FTT_0533c; FTL_0985	1.5	-1.82	No Change at 4/8 hr.	Not essential (*38*), Essential in Fn replication in U937 cells (*49*)	Not essential (*38*) Essential Schu S4 (*41*)	Essential (*38, 41*)
grxB	Glutaredoxin B	FTN_1033; FTT_0650c; FTL_0923.	1.2	-1.65	No change at 4 hr/ Up Regulated at 8 hr.	Not essential (*38*)	Not essential (*38*)	No data
grx3/C #/	Glutaredoxin reductase	FTN_0983; FTT_0532c; FTL_0984.	1.6	No data	No Change at 4/8 hr.	Not essential LVS (*38*)	Not essential (*38*) Essential Schu S4 (*41*)	Essential (*41*)
gloA $	Lacto-glutathione lyase	FTN_1231; FTT_1212c; FTL_0732.	1.6	No data	Down Regulated at 4 hr/ No change at 8 hr.	Not essential (*38*), Essential in Fn replication in U937 and S2 cells (*49*)	Not essential (*38*)	No effect reported (*41*)
pepA **/$*	Amino peptidase A	FTN_0660; FTT_1318c; FTL_1479.	1.4	-2.51	No Change at 4/8 hr.	Essential (*38*) (-1.36 log2FC)	Not essential (*38*)	Essential (*41*)
pepB	Amino peptidase B	FTN_0780; FTT_1094c; FTL_1108 (DA46-1685).	1.5	No data	No Change at 4/8 hr.	Not essential (*38*)	Not essential (*38*)	No effect reported (*41*)
pepN	Amino peptidase N	FTN_1798; FTT_1793c; FTL_1956.	2.4	-1.72	No Change at 4/8 hr.	Not essential (*38*)	Not essential (*38*)	No effect reported (*41*)
trxA1 $	Thioredoxin A1	FTN_1415; FTT_1445; FTL_0611.	1.1	No data	No Change at 4/8 hr.	Essential (*38, 57*) (-1.69 log2FC).	Not essential (*38*)	No effect reported (*41*)

trxA2	Thioredoxin A2	FTN_0856; FTT_0976; FTL_1224.	No data	No data	Up regulated at 4 (2.83x) and 8 hr (6.33x). **	Not essential (*38*)	Not essential (*38*)	No effect reported (*41*)
trxB/ trxR	Thioredoxin reductase	FTN_0580; FTT_0489c; FTL_1571.	1.3	No data	No Change at 4/8 hr.	Not essential (*38*)	Not essential (*38*)	No effect reported (*41*)
fupA $	GSH porin (*85*).	FTN_0444; FTT_0918; FTL_0439.	0.6	-2.05	Up Regulated at 4 hr (1.97) but Down at 8 hr (0.8).	Essential (*38*) (-1.55 log2FC). Essential in U937 cells (*49*)	Essential in LVS (*38*)	No effect reported (*41*)
ngtA	MFS superfamily (proposed GSH transporter) (*85*).	FTN_1011; FTT_0671; FTL_0946	No data	1.5	No data	Not essential (*38*),	Not essential (*38*)	No effect reported (*41*)
Cgc1 or cgaA	CobB/CobQ-like glutamine amidotransferase domain protein (proposed GSH breakdown) (*85*).	FTN_0435; FTT_0909; FTL_0429	No data	No data	Down Regulated at 4 hr (0.37) & 8 hr (0.36).	Not essential (*38*). Essential in Fn replication in S2 cells (*49*)	Essential in LVS (*38*)	No effect reported (*41*)

Supplemental Figures and Tables:

Figure S1: Glutathione metabolism pathway in *Francisella tularensis* subsp. *tularensis* Schu S4 according to KEGG. The green highlighted boxes illustrate the enzymes that are present in the *Francisella* Schu S4 genome in the KEGG pathway of GSH metabolism (FTU00480). If not named, the E.C. number is listed – please see Table 3 for those numbers. Specifically, boxes for *ggt*, *gor*, *glutathione peroxidase (GSHPx/GPx)*, *gshA* and *gshB* proteins can be seen on the left perimeter of this pathway. Boxes for *pepN*, *pepA* and *pepB* are clustered here as well. Additional genes that do not have homologs in Francisella are shown in white boxes. Image from: https://www.kegg.jp/kegg-bin/show_pathway?ftu00480

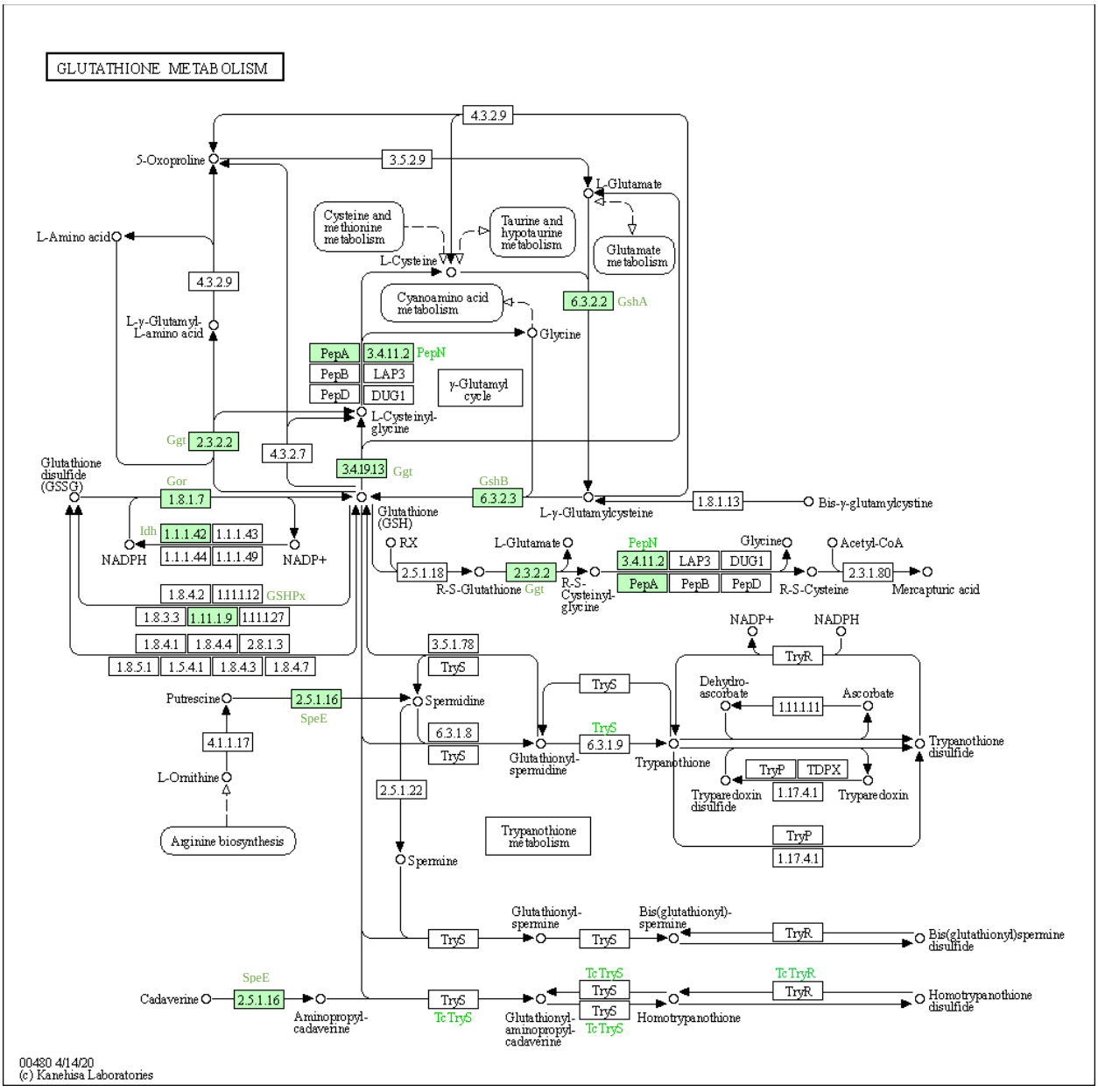


Figure S2: Low MW Thiol detection in *Francisella* samples. Determination of LMW Thiols in *Francisella* cell pellet samples. Chromatogram data of bacterial pellets of *F. novicida* U112 and *F. philomiragia* 25017 under reduced thiol conditions (b, d) or NEM control conditions (c, e) compared to standard controls shown in (a). **Method:** Two strains of *Francisella* were grown overnight in tryptic soy broth containing 0.1% CSH (TSBC) broth for 18 hours. LMW thiols were measured by HPLC analysis of fluorescent thiol adducts with monobromobimane as described previously (*33*) using a Waters HPLC instrument. The amount of thiol was normalized by dry weight of sample.

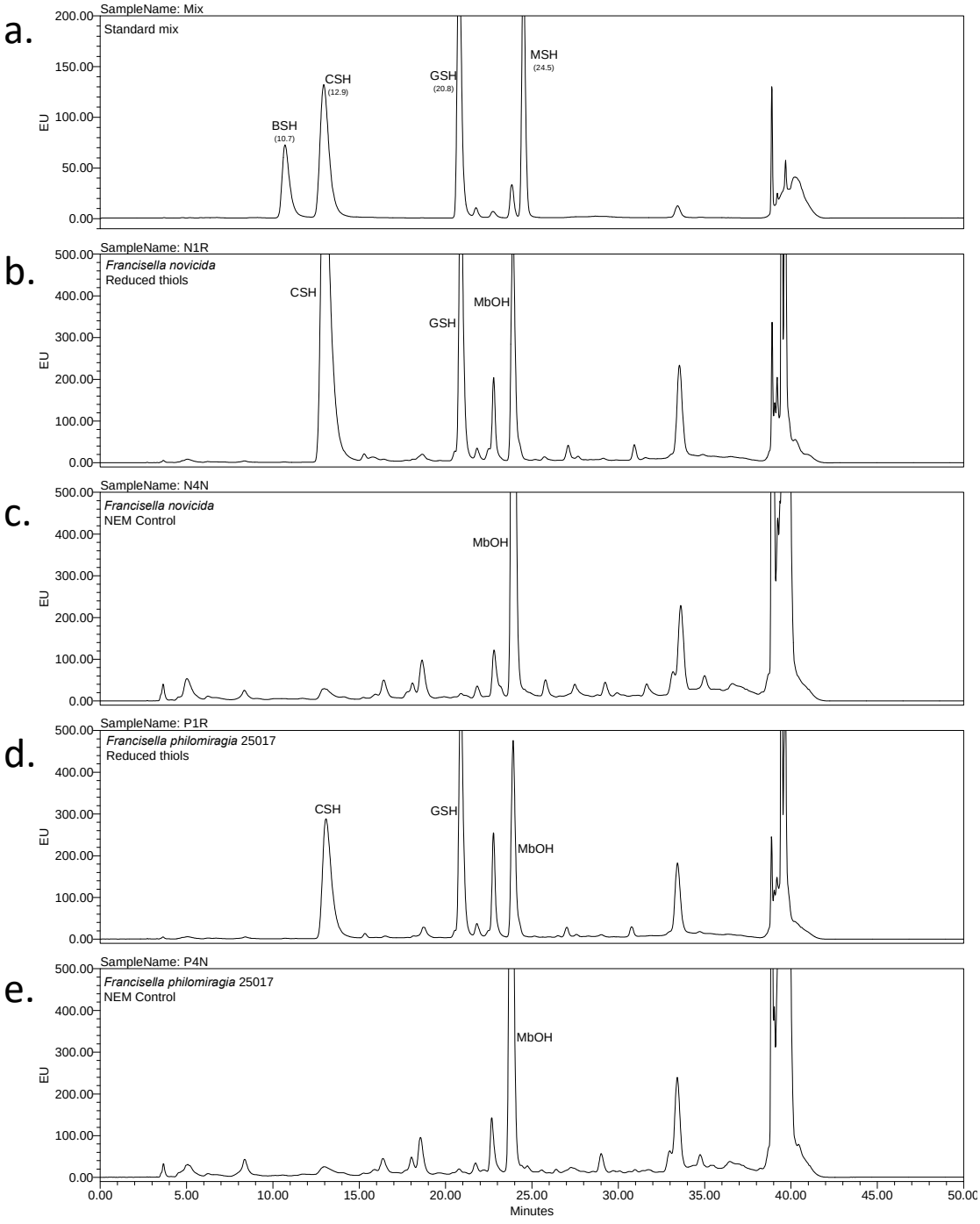


Table S1. Analysis of chromatogram data. Quantitative results of chromatograms of LMR thiol analysis of *Francisella* pellets presented in **Figure S2**.

Sample	Sample thiol (nmol total) Ave ± SD	Amount of thiol (µmole/g) Ave ± SD
Francisella novicida		
CSH	81.92 ± 2.01	7.89 ± 0.51
GSH	31.11 ± 0.79	3.00 ± 0.20
Francisella philomiragia		
CSH	27.05 ± 0.61	3.19 ± 0.12
GSH	25.90 ± 0.71	3.05 ± 0.09

References:

1. J. Ellis, P. C. Oyston, M. Green, R. W. Titball, Tularemia. *Clin Microbiol Rev* **15**, 631-646 (2002).
2. K. Alkhuder, K. L. Meibom, I. Dubail, M. Dupuis, A. Charbit, Glutathione provides a source of cysteine essential for intracellular multiplication of Francisella tularensis. *PLoS Pathog* **5**, e1000284 (2009).
3. L. C. Kingry, J. M. Petersen, Comparative review of Francisella tularensis and Francisella novicida. *Front Cell Infect Microbiol* **4**, 35 (2014).
4. L. Rohmer *et al.*, Comparison of Francisella tularensis genomes reveals evolutionary events associated with the emergence of human pathogenic strains. *Genome Biol* **8**, R102 (2007).
5. P. Keim, A. Johansson, D. M. Wagner, Molecular epidemiology, evolution, and ecology of Francisella. *Ann N Y Acad Sci* **1105**, 30-66 (2007).
6. M. K. McLendon, M. A. Apicella, L. A. Allen, Francisella tularensis: taxonomy, genetics, and Immunopathogenesis of a potential agent of biowarfare. *Annu Rev Microbiol* **60**, 167-185 (2006).
7. T. Guina *et al.*, MglA regulates Francisella tularensis subsp. novicida (Francisella novicida) response to starvation and oxidative stress. *J Bacteriol* **189**, 6580-6586 (2007).
8. M. Rawat, J. A. Maupin-Furlow, Redox and Thiols in Archaea. *Antioxidants (Basel)* **9**, (2020).
9. S. Siddaramappa, J. F. Challacombe, J. M. Petersen, S. Pillai, C. R. Kuske, Genetic diversity within the genus Francisella as revealed by comparative analyses of the genomes of two North American isolates from environmental sources. *BMC Genomics* **13**, 422 (2012).
10. J. Mikalsen *et al.*, Virulence and pathogenicity of Francisella philomiragia subsp. noatunensis for Atlantic cod, Gadus morhua L., and laboratory mice. *J Fish Dis* **32**, 377-381 (2009).
11. C. E. Bradburne *et al.*, Temporal transcriptional response during infection of type II alveolar epithelial cells with Francisella tularensis live vaccine strain (LVS) supports a general host suppression and bacterial uptake by macropinocytosis. *J Biol Chem* **288**, 10780-10791 (2013).
12. L. D. Anthony, R. D. Burke, F. E. Nano, Growth of Francisella spp. in rodent macrophages. *Infect Immun* **59**, 3291-3296 (1991).
13. A. Chong, J. Celli, The francisella intracellular life cycle: toward molecular mechanisms of intracellular survival and proliferation. *Front Microbiol* **1**, 138 (2010).
14. K. Gustafsson, Growth and survival of four strains of Francisella tularensis in a rich medium preconditioned with Acanthamoeba palestinensis. *Can J Microbiol* **35**, 1100-1104 (1989).
15. H. Abd, T. Johansson, I. Golovliov, G. Sandstrom, M. Forsman, Survival and growth of Francisella tularensis in Acanthamoeba castellanii. *Appl Environ Microbiol* **69**, 600-606 (2003).
16. C. M. Lauriano *et al.*, MglA regulates transcription of virulence factors necessary for Francisella tularensis intraamoebae and intramacrophage survival. *Proc Natl Acad Sci U S A* **101**, 4246-4249 (2004).

17. S. H. El-Etr *et al.*, Francisella tularensis type A strains cause the rapid encystment of Acanthamoeba castellanii and survive in amoebal cysts for three weeks postinfection. *Appl Environ Microbiol* **75**, 7488-7500 (2009).
18. A. B. Verhoeven, M. W. Durham-Colleran, T. Pierson, W. T. Boswell, M. L. Van Hoek, Francisella philomiragia biofilm formation and interaction with the aquatic protist Acanthamoeba castellanii. *Biol Bull* **219**, 178-188 (2010).
19. M. L. van Hoek, Biofilms: an advancement in our understanding of Francisella species. *Virulence* **4**, 833-846 (2013).
20. H. Y. Buse, F. W. Schaefer, III, E. W. Rice, Enhanced survival but not amplification of Francisella spp. in the presence of free-living amoebae. *Acta Microbiol Immunol Hung* **64**, 17-36 (2017).
21. Y. Brenz, H. C. Winther-Larsen, M. Hagedorn, Expanding Francisella models: Pairing up the soil amoeba Dictyostelium with aquatic Francisella. *Int J Med Microbiol* **308**, 32-40 (2018).
22. K. Koppen *et al.*, Screen for fitness and virulence factors of Francisella sp. strain W12-1067 using amoebae. *Int J Med Microbiol* **309**, 151341 (2019).
23. I. Kelava *et al.*, Optimisation of External Factors for the Growth of Francisella novicida within Dictyostelium discoideum. *Biomed Res Int* **2020**, 6826983 (2020).
24. K. L. Meibom, A. Charbit, Francisella tularensis metabolism and its relation to virulence. *Front Microbiol* **1**, 140 (2010).
25. A. Sjostedt, Intracellular survival mechanisms of Francisella tularensis, a stealth pathogen. *Microbes Infect* **8**, 561-567 (2006).
26. M. Barel, E. Ramond, G. Gesbert, A. Charbit, The complex amino acid diet of Francisella in infected macrophages. *Front Cell Infect Microbiol* **5**, 9 (2015).
27. C. N. Propst *et al.*, Francisella philomiragia Infection and Lethality in Mammalian Tissue Culture Cell Models, Galleria mellonella, and BALB/c Mice. *Front Microbiol* **7**, 696 (2016).
28. J. Ziveri, M. Barel, A. Charbit, Importance of Metabolic Adaptations in Francisella Pathogenesis. *Front Cell Infect Microbiol* **7**, 96 (2017).
29. S. Steele *et al.*, Francisella tularensis harvests nutrients derived via ATG5-independent autophagy to support intracellular growth. *PLoS Pathog* **9**, e1003562 (2013).
30. R. L. Markley *et al.*, Macrophage Selenoproteins Restrict Intracellular Replication of Francisella tularensis and Are Essential for Host Immunity. *Front Immunol* **12**, 701341 (2021).
31. M. Santic, Y. Abu Kwaik, Nutritional virulence of Francisella tularensis. *Front Cell Infect Microbiol* **3**, 112 (2013).
32. G. Gesbert *et al.*, Importance of branched-chain amino acid utilization in Francisella intracellular adaptation. *Infect Immun* **83**, 173-183 (2015).
33. G. L. Newton *et al.*, Bacillithiol is an antioxidant thiol produced in Bacilli. *Nat Chem Biol* **5**, 625-627 (2009).
34. G. L. Newton, M. Rawat, N-methyl-bacillithiol, a Novel Thiol from Anaerobic Bacteria. *mBio* **10**, (2019).
35. L. Masip, K. Veeravalli, G. Georgiou, The many faces of glutathione in bacteria. *Antioxid Redox Signal* **8**, 753-762 (2006).

36. E. Ramond *et al.*, Glutamate utilization couples oxidative stress defense and the tricarboxylic acid cycle in Francisella phagosomal escape. *PLoS Pathog* **10**, e1003893 (2014).
37. J. Binesse, H. Lindgren, L. Lindgren, W. Conlan, A. Sjostedt, Roles of reactive oxygen species-degrading enzymes of Francisella tularensis SCHU S4. *Infect Immun* **83**, 2255-2263 (2015).
38. K. M. Ramsey *et al.*, Tn-Seq reveals hidden complexity in the utilization of host-derived glutathione in Francisella tularensis. *PLoS Pathog* **16**, e1008566 (2020).
39. S. D. Copley, J. K. Dhillon, Lateral gene transfer and parallel evolution in the history of glutathione biosynthesis genes. *Genome Biol* **3**, research0025 (2002).
40. L. A. Gallagher *et al.*, A comprehensive transposon mutant library of Francisella novicida, a bioweapon surrogate. *Proc Natl Acad Sci U S A* **104**, 1009-1014 (2007).
41. P. M. Ireland *et al.*, Global Analysis of Genes Essential for Francisella tularensis Schu S4 Growth In Vitro and for Fitness during Competitive Infection of Fischer 344 Rats. *J Bacteriol* **201**, (2019).
42. E. S. Arner, A. Holmgren, Physiological functions of thioredoxin and thioredoxin reductase. *Eur J Biochem* **267**, 6102-6109 (2000).
43. M. Russel, A. Holmgren, Construction and characterization of glutaredoxin-negative mutants of Escherichia coli. *Proc Natl Acad Sci U S A* **85**, 990-994 (1988).
44. H. C. May *et al.*, Thioredoxin-A is a virulence factor and mediator of the type IV pilus system in Acinetobacter baumannii. *PLoS One* **14**, e0218505 (2019).
45. J. Norambuena, Y. Wang, T. Hanson, J. M. Boyd, T. Barkay, Low-Molecular-Weight Thiols and Thioredoxins Are Important Players in Hg(II) Resistance in Thermus thermophilus HB27. *Appl Environ Microbiol* **84**, (2018).
46. C. Cheng *et al.*, Thioredoxin A Is Essential for Motility and Contributes to Host Infection of Listeria monocytogenes via Redox Interactions. *Front Cell Infect Microbiol* **7**, 287 (2017).
47. T. D. Wehrly *et al.*, Intracellular biology and virulence determinants of Francisella tularensis revealed by transcriptional profiling inside macrophages. *Cell Microbiol* **11**, 1128-1150 (2009).
48. R. Asare, C. Akimana, S. Jones, Y. Abu Kwaik, Correction to: Molecular bases of proliferation of Francisella tularensis in arthropod vectors. *Environ Microbiol* **13**, 3311-3311 (2011).
49. R. Asare, Y. Abu Kwaik, Corrections to: Molecular complexity orchestrates modulation of phagosome biogenesis and escape to the cytosol of macrophages by Francisella tularensis. *Environ Microbiol* **13**, 3310-3310 (2011).
50. D. S. Weiss *et al.*, In vivo negative selection screen identifies genes required for Francisella virulence. *Proc Natl Acad Sci U S A* **104**, 6037-6042 (2007).
51. K. L. Meibom, A. Charbit, The unraveling panoply of Francisella tularensis virulence attributes. *Curr Opin Microbiol* **13**, 11-17 (2010).
52. A. Read, S. J. Vogl, K. Hueffer, L. A. Gallagher, G. M. Happ, Francisella genes required for replication in mosquito cells. *J Med Entomol* **45**, 1108-1116 (2008).

53. O. Carmel-Harel, G. Storz, Roles of the glutathione- and thioredoxin-dependent reduction systems in the Escherichia coli and saccharomyces cerevisiae responses to oxidative stress. *Annu Rev Microbiol* **54**, 439-461 (2000).
54. J. Lu, A. Holmgren, The thioredoxin antioxidant system. *Free Radic Biol Med* **66**, 75-87 (2014).
55. Z. Ma *et al.*, Elucidation of a mechanism of oxidative stress regulation in Francisella tularensis live vaccine strain. *Mol Microbiol* **101**, 856-878 (2016).
56. H. C. May *et al.*, Thioredoxin Modulates Cell Surface Hydrophobicity in Acinetobacter baumannii. *Front Microbiol* **10**, 2849 (2019).
57. P. S. Kraemer *et al.*, Genome-wide screen in Francisella novicida for genes required for pulmonary and systemic infection in mice. *Infect Immun* **77**, 232-244 (2009).
58. Z. Ma, M. Higgs, M. Alqahtani, C. S. Bakshi, M. Malik, ThioredoxinA1 Controls the Oxidative Stress Response of Francisella tularensis Live Vaccine Strain (LVS). *J Bacteriol* **204**, e0008222 (2022).
59. P. J. Murray, T. A. Wynn, Protective and pathogenic functions of macrophage subsets. *Nat Rev Immunol* **11**, 723-737 (2011).
60. M. Honn, H. Lindgren, G. K. Bharath, A. Sjostedt, Lack of OxyR and KatG Results in Extreme Susceptibility of Francisella tularensis LVS to Oxidative Stress and Marked Attenuation In vivo. *Front Cell Infect Microbiol* **7**, 14 (2017).
61. M. Honn, H. Lindgren, A. Sjostedt, The role of MglA for adaptation to oxidative stress of Francisella tularensis LVS. *BMC Microbiol* **12**, 14 (2012).
62. J. Lenco, I. Pavkova, M. Hubalek, J. Stulik, Insights into the oxidative stress response in Francisella tularensis LVS and its mutant DeltaiglC1+2 by proteomics analysis. *FEMS Microbiol Lett* **246**, 47-54 (2005).
63. Z. Ma *et al.*, Stringent response governs the oxidative stress resistance and virulence of Francisella tularensis. *PLoS One* **14**, e0224094 (2019).
64. D. Marghani *et al.*, An AraC/XylS Family Transcriptional Regulator Modulates the Oxidative Stress Response of Francisella tularensis. *J Bacteriol* **203**, e0018521 (2021).
65. H. Lindgren *et al.*, Resistance of Francisella tularensis strains against reactive nitrogen and oxygen species with special reference to the role of KatG. *Infect Immun* **75**, 1303-1309 (2007).
66. A. Alharbi *et al.*, Role of peroxiredoxin of the AhpC/TSA family in antioxidant defense mechanisms of Francisella tularensis. *PLoS One* **14**, e0213699 (2019).
67. L. B. Poole, Bacterial defenses against oxidants: mechanistic features of cysteine-based peroxidases and their flavoprotein reductases. *Arch Biochem Biophys* **433**, 240-254 (2005).
68. C. F. Djuika *et al.*, Plasmodium falciparum antioxidant protein as a model enzyme for a special class of glutaredoxin/glutathione-dependent peroxiredoxins. *Biochim Biophys Acta* **1830**, 4073-4090 (2013).
69. M. L. Reniere, Reduce, Induce, Thrive: Bacterial Redox Sensing during Pathogenesis. *J Bacteriol* **200**, (2018).
70. S. Fourquet, M. E. Huang, B. D'Autreaux, M. B. Toledano, The dual functions of thiol-based peroxidases in H2O2 scavenging and signaling. *Antioxid Redox Signal* **10**, 1565-1576 (2008).

71. V. T. Maulik, S. L. Jennifer, J. S. Teruna, The Role of Thiols and Disulfides on Protein Stability. *Current Protein & Peptide Science* **10**, 614-625 (2009).
72. Q. N. Tung, N. Linzner, V. V. Loi, H. Antelmann, Application of genetically encoded redox biosensors to measure dynamic changes in the glutathione, bacillithiol and mycothiol redox potentials in pathogenic bacteria. *Free Radic Biol Med* **128**, 84-96 (2018).
73. T. Pierson *et al.*, Proteomic characterization and functional analysis of outer membrane vesicles of Francisella novicida suggests possible role in virulence and use as a vaccine. *J Proteome Res* **10**, 954-967 (2011).
74. P. Spidlova, P. Stojkova, A. Sjostedt, J. Stulik, Control of Francisella tularensis Virulence at Gene Level: Network of Transcription Factors. *Microorganisms* **8**, (2020).
75. K. Kadzhaev *et al.*, Identification of genes contributing to the virulence of Francisella tularensis SCHU S4 in a mouse intradermal infection model. *PLoS One* **4**, e5463 (2009).
76. F. Aslund, J. Beckwith, Bridge over troubled waters: sensing stress by disulfide bond formation. *Cell* **96**, 751-753 (1999).
77. J. M. Dubbs, S. Mongkolsuk, Peroxide-sensing transcriptional regulators in bacteria. *J Bacteriol* **194**, 5495-5503 (2012).
78. M. Hillion, H. Antelmann, Thiol-based redox switches in prokaryotes. *Biol Chem* **396**, 415-444 (2015).
79. A. P. Wrench, C. L. Gardner, C. F. Gonzalez, G. L. Lorca, Identification of a small molecule that modifies MglA/SspA interaction and impairs intramacrophage survival of Francisella tularensis. *PLoS One* **8**, e54498 (2013).
80. J. C. Charity, L. T. Blalock, M. M. Costante-Hamm, D. L. Kasper, S. L. Dove, Small molecule control of virulence gene expression in Francisella tularensis. *PLoS Pathog* **5**, e1000641 (2009).
81. B. L. Bell, N. P. Mohapatra, J. S. Gunn, Regulation of virulence gene transcripts by the Francisella novicida orphan response regulator PmrA: role of phosphorylation and evidence of MglA/SspA interaction. *Infect Immun* **78**, 2189-2198 (2010).
82. J. Wong, Y. Chen, Y. H. Gan, Host Cytosolic Glutathione Sensing by a Membrane Histidine Kinase Activates the Type VI Secretion System in an Intracellular Bacterium. *Cell Host Microbe* **18**, 38-48 (2015).
83. M. Hall *et al.*, Structural basis for glutathione-mediated activation of the virulence regulatory protein PrfA in Listeria. *Proc Natl Acad Sci U S A* **113**, 14733-14738 (2016).
84. M. Deponte, Glutathione catalysis and the reaction mechanisms of glutathione-dependent enzymes. *Biochim Biophys Acta* **1830**, 3217-3266 (2013).
85. Y. Wang *et al.*, Discovery of a glutathione utilization pathway in Francisella that shows functional divergence between environmental and pathogenic species. *Cell Host Microbe* **31**, 1359-1370 e1357 (2023).
86. J. W. Ku, Y. H. Gan, Modulation of bacterial virulence and fitness by host glutathione. *Curr Opin Microbiol* **47**, 8-13 (2019).
87. T. M. Maier *et al.*, Identification of Francisella tularensis Himar1-based transposon mutants defective for replication in macrophages. *Infect Immun* **75**, 5376-5389 (2007).
88. A. Qin, B. J. Mann, Identification of transposon insertion mutants of Francisella tularensis tularensis strain Schu S4 deficient in intracellular replication in the hepatic cell line HepG2. *BMC Microbiol* **6**, 69 (2006).

89. I. Castellano, A. Merlino, gamma-Glutamyltranspeptidases: sequence, structure, biochemical properties, and biotechnological applications. *Cell Mol Life Sci* **69**, 3381-3394 (2012).
90. K. M. Holland *et al.*, Differential Growth of Francisella tularensis, Which Alters Expression of Virulence Factors, Dominant Antigens, and Surface-Carbohydrate Synthases, Governs the Apparent Virulence of Ft SchuS4 to Immunized Animals. *Front Microbiol* **8**, 1158 (2017).
91. Z. W. Bent *et al.*, Use of a capture-based pathogen transcript enrichment strategy for RNA-Seq analysis of the Francisella tularensis LVS transcriptome during infection of murine macrophages. *PLoS One* **8**, e77834 (2013).
92. M. S. Pittman, H. C. Robinson, R. K. Poole, A bacterial glutathione transporter (Escherichia coli CydDC) exports reductant to the periplasm. *J Biol Chem* **280**, 32254-32261 (2005).
93. M. C. Walker, W. A. van der Donk, The many roles of glutamate in metabolism. *J Ind Microbiol Biotechnol* **43**, 419-430 (2016).
94. A. L. Murch, P. J. Skipp, P. L. Roach, P. C. F. Oyston, Whole genome transcriptomics reveals global effects including up-regulation of Francisella pathogenicity island gene expression during active stringent response in the highly virulent Francisella tularensis subsp. tularensis SCHU S4. *Microbiology (Reading)* **163**, 1664-1679 (2017).
95. A. Raghunathan, S. Shin, S. Daefler, 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).
96. M. L. Reniere *et al.*, Glutathione activates virulence gene expression of an intracellular pathogen. *Nature* **517**, 170-173 (2015).
97. S. Brouwer *et al.*, Streptococcus pyogenes Hijacks Host Glutathione for Growth and Innate Immune Evasion. *mBio* **13**, e0067622 (2022).
98. B. Vergauwen, J. Elegheert, A. Dansercoer, B. Devreese, S. N. Savvides, Glutathione import in Haemophilus influenzae Rd is primed by the periplasmic heme-binding protein HbpA. *Proc Natl Acad Sci U S A* **107**, 13270-13275 (2010).
99. B. Vergauwen, F. Pauwels, M. Vaneechoutte, J. J. Van Beeumen, Exogenous glutathione completes the defense against oxidative stress in Haemophilus influenzae. *J Bacteriol* **185**, 1572-1581 (2003).
100. P. Larsson *et al.*, The complete genome sequence of Francisella tularensis, the causative agent of tularemia. *Nat Genet* **37**, 153-159 (2005).
101. R. D. Barabote *et al.*, Complete genome sequence of Francisella tularensis subspecies holarctica FTNF002-00. *PLoS One* **4**, e7041 (2009).
102. J. Su *et al.*, Genome-wide identification of Francisella tularensis virulence determinants. *Infect Immun* **75**, 3089-3101 (2007).
103. A. Meireles Dde, T. G. Alegria, S. V. Alves, C. R. Arantes, L. E. Netto, A 14.7 kDa protein from Francisella tularensis subsp. novicida (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).

