



Diverse roles of low-molecular weight thiol GSH in *Francisella*'s virulence, location sensing and GSH-stealing from host

Monique L. van Hoek^{a,*}, Alexander Marchesani^b, Mamta Rawat^c

^a School of Systems Biology, George Mason University, Manassas, VA, United States

^b Department of Biology, George Mason University, Manassas, VA, United States

^c Biology Department, California State University, Fresno, CA, United States

ARTICLE INFO

Keywords:

Glutathione
GSH
Bacterial GSH-cleaving enzymes
Glutamate importer
GSH exporter
Francisella
Thioredoxin
Glutaredoxin

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 *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.

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 (Ellis et al., 2002), 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.

* Corresponding author at: 10910 George Mason Circle MS1H8, George Mason University, Manassas, VA 20110.

E-mail address: mvanhoek@gmu.edu (M.L. van Hoek).

<https://doi.org/10.1016/j.crmicr.2023.100218>

Available online 20 December 2023

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Francisella subspecies and intracellular lifestyle

The bacterium *F. tularensis* has 4 major subspecies (Ellis et al., 2002; Alkhuder et al., 2009; Kingry and Petersen, 2014). *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 (Ellis et al., 2002). This includes the strain Schu S4 which is commonly studied. The subspecies *holarctica* (Type B) is a less virulent form commonly found in northern Eurasian countries, such as Russia, Sweden, and Norway, as well as Japan, and causes most of the global cases of human tularemia (Ellis et al., 2002; Kingry and Petersen, 2014; Rohmer et al., 2007; Keim et al., 2007). 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 % nucleotide identity) and is lethal to mice (Rohmer et al., 2007). *F. tularensis* subspecies *mediasiatica* is a less common human and mammalian pathogen found in central Asia (McLendon et al., 2006; Guina et al., 2007). 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 (Guina et al., 2007; Rawat and Maupin-Furlow, 2020; Siddaramappa et al., 2012). This strain is also commonly employed as a Biosafety Level 2 experimental model for the more virulent strains, along with the Live Vaccine strain (Kingry and Petersen, 2014). There are several other environmental species in the genus, including *F. philomiragia* and *F. noatunensis* (Mikalsen et al., 2009). *Francisella* species have been reported to infect over 250 species of animals, from birds to fish, cats, dogs, rodents, amoeba and humans (Keim et al., 2007), 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 (Ellis et al., 2002) or non-phagocytic cells (Bradburne et al., 2013) 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 (Anthony et al., 1991), 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 (Chong and Celli, 2010). *Francisella* in the environment may or may not have this intracellular lifestyle, as it has been reported in water, mud, soil, rodent droppings and rodent carcasses (Ellis et al., 2002), although it has been detected within single-cellular eukaryotic aquatic amoeba (Gustafsson, 1989; Abd et al., 2003; Lauriano et al., 2004; El-Etr et al., 2009; Verhoeven et al., 2010; van Hoek, 2013; Buse et al., 2017; Brenz et al., 2018; Koppen et al., 2019; Kelava et al., 2020).

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 its *in vitro* laboratory growth (Alkhuder et al., 2009; Meibom and Charbit, 2010; Sjøstedt, 2006). *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 (Meibom and Charbit, 2010; Barel et al., 2015). This bacterium requires a large exogenously supplied pool of these amino acids, especially cysteine (CSH) for growth *in vitro* and for the robust intracellular proliferation that occurs in the cytosol of host cells (Meibom and Charbit, 2010; Barel et al., 2015) where it must obtain these amino acids from the host during infection.

Nutritional challenges of intracellular growth for Francisella

F. tularensis is *facultatively* intracellular in eukaryotic cells, from amoeba to insect cells and from to mammalian macrophages to hepatocytes and epithelial cells (van Hoek, 2013; Propst et al., 2016). Like *Rickettsia*, *Shigella* and *Listeria*, *Francisella* bacteria rapidly escape the phagosome to replicate to extremely high numbers in the host cytoplasm (Ellis et al., 2002; Ziveri et al., 2017; Steele et al., 2013). This 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 (Chong and Celli, 2010; Markley et al., 2021; Santic and Abu Kwaik, 2013). 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 (Bradburne et al., 2013), 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 (Ellis et al., 2002; McLendon et al., 2006). 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 (Steele et al., 2013). 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 (Markley et al., 2021), which is the basis of "nutritional immunity" (Santic and Abu Kwaik, 2013). Recent studies in *Francisella* metabolism have begun to explore this important topic of nutritional interaction (Meibom and Charbit, 2010; Barel et al., 2015; Ziveri et al., 2017; Steele et al., 2013; Santic and Abu Kwaik, 2013; Gesbert et al., 2015), especially with regards to glutathione (GSH) (Fig. 1A-F).

Glutathione is the major LMW thiol in Francisella

Glutathione (GSH) is a non-ribosomal thiol tripeptide, (γ -L-Glutamyl-L-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 h (van Hoek and Rawat, *personal communication*). LMW thiols were measured by HPLC analysis of fluorescent thiol adducts with monobromobimane (Newton et al., 2009). The amount of thiol was normalized by dry weight of sample. The results (Table 2) identified GSH at approximately 3 μ mol/g dry weight and CSHCSH at high levels as well. The levels of CSH in *Francisella* were high compared to other bacteria (Newton and Rawat, 2019) 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 (Masip et al., 2006; Ramond et al., 2014). Like most gamma proteobacteria, *Francisella* has genes for both *gshA* and *gshB* (Table 1, Fig. 1A,B) (Alkhuder et al., 2009; Ramond et al., 2014; Binesse et al., 2015; Ramsey et al., 2020). 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 (γ GC) with glycine to generate GSH (Copley and Dhillon, 2002). Both GshA and GshB proteins are in the *Francisella* cytoplasm (Alkhuder et al.,

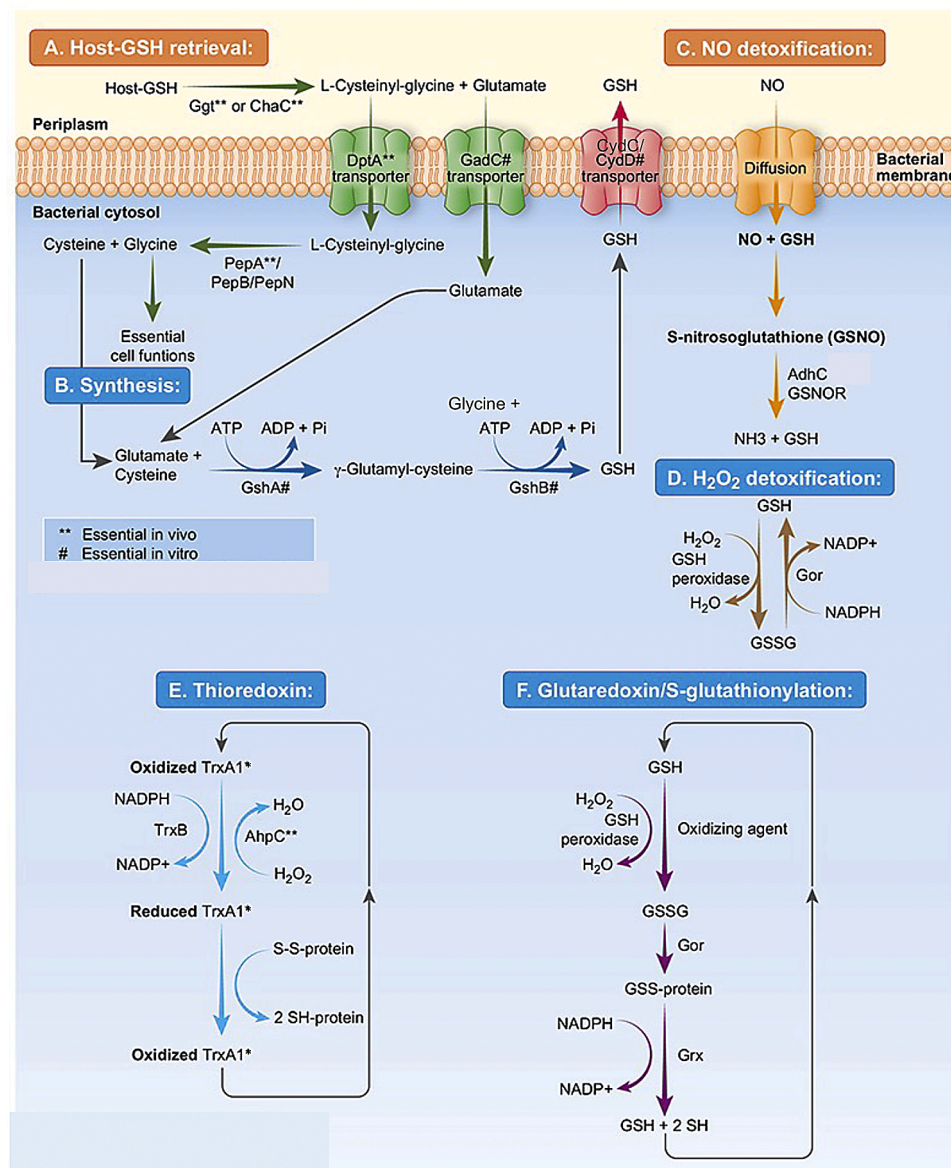


Fig. 1. Low MW thiols in *Francisella*. Host-derived or exogenous GSH can be transported into the periplasm (Fig. 1A), potentially by FupA GSH porin in the OM (not shown). From within the periplasm, in the presence of high levels of exogenous GSH, such as in the cytosol of a macrophage or from a host, 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) (Fig. 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 (Fig. 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 (Fig. 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) (Fig. 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 (Fig. 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 (Fig. 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. (Fig. 1E). Symbols: ** essential in vivo, # essential in vitro.

2009; Ramsey et al., 2020). 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. (2007). Similarly, *gshA* and *gshB* were found to be essential for *F. tularensis* Schu S4 growth *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	<i>Francisella tularensis</i> subsp. <i>tularensis</i> (strain Schu S4)	<i>Francisella tularensis</i> subsp. <i>novicida</i> (strain U112)	<i>Francisella tularensis</i> subsp. <i>holarctica</i> (strain LVS)
Intra-bacterial Glutathione/GSH biosynthesis (cytoplasmic) (Fig. 1A)			
Glutamate-CSH ligase, <i>gshA</i> # [EC:6.3.2.2] (K01919)	FTT_0367c	FTN_0277	FTL_1304
Glutathione synthetase, <i>gshB</i> # [EC:6.3.2.3] (K01920)	FTT_0926	FTN_0804	FTL_1284
Periplasmic GSH Cleavage to L-cysteinyl glycine (di-peptide) (Fig. 1A)			
γ -Glutamyl transpeptidase, <i>ggt</i> ** [EC:2.3.2.2, 3.4.19.13] (K00681)	FTT_1181c	FTN_1159	FTL_0766
γ -glutamyl-cyclotransferase (GGCT), <i>chaC</i> ** [EC:4.3.2.9]	FTT_0509c	FTN_0599	FTL_1548
Di-peptide transport from Periplasm to Cytosol (Fig. 1A)			
<i>dptA</i> ** amino-acid/di-peptide transporter family protein; (K03305) (di-or tripeptide:H ⁺ symporter)	FTT_0953c	FTN_0832	FTL_1251
<i>gadC</i> # glutamate: γ -aminobutyrate (GABA) exchanger (K20265)	FTT_0480c	FTN_0571	FTL_1583
GSH Export from Cytosol to Periplasm (Fig. 1A)			
CSH/GSH ABC transporter permease/ATP-binding protein <i>cydD</i> # (K16013)/ <i>cydC</i> (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, <i>pepA</i> ** [EC:3.4.11.1] (K01255)	FTT_1318c	FTN_0660	FTL_1479
cytosol leucyl Aminopeptidase B, <i>pepB</i> [EC:3.4.11.1] (K01255)	FTT_1094c	FTN_0780	FTL_1108 (DA46-1685)
membrane alanyl Aminopeptidase N, <i>pepN</i> ; [EC:3.4.11.2] (K01256)	FTT_1793c	FTN_1768	FTL_1956
Intra-bacterial Glutathione/GSH biosynthesis (cytoplasmic) (Fig. 1B)			
Glutamate-CSH ligase, <i>gshA</i> # [EC:6.3.2.2] (K01919)	FTT_0367c	FTN_0277	FTL_1304
Glutathione synthetase, <i>gshB</i> # [EC:6.3.2.3] (K01920)	FTT_0926	FTN_0804	FTL_1284
NO detoxification (Fig. 1C)			
<i>adhC</i>	–	FTN_0409	–
H₂O₂ detoxification (Fig. 1D)			
Glutathione reductase, <i>gor</i> [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 <i>Francisella</i> (Protein disulfide isomerases). Thioredoxins (Fig. 1E)			
<i>trxA1</i> #; thioredoxin (K03671)	FTT_1445	FTN_1415	FTL_1224
<i>trxA2</i> ; thioredoxin (K03671)	FTT_0976	FTN_0856	FTL_0611
<i>trxB/trxR</i> ; thioredoxin reductase (K00384)	FTT_0489c	FTN_0580	FTL_1571
Glutaredoxins (Fig. 1F)			

Table 1 (continued)

Gene	<i>Francisella tularensis</i> subsp. <i>tularensis</i> (strain Schu S4)	<i>Francisella tularensis</i> subsp. <i>novicida</i> (strain U112)	<i>Francisella tularensis</i> subsp. <i>holarctica</i> (strain LVS)
<i>grxA</i> #; glutaredoxin 1 (K03674)	FTT_0533c	FTN_0982	FTL_0985
<i>grxB</i> ; glutaredoxin 2 (K03675)	FTT_0650c	FTN_1033	FTL_0923
glutaredoxin-like protein <i>grxC</i> #; 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	FTL_0984
<i>grxD</i> ; monothiol glutaredoxin (K07390)	FTT_0067c	FTN_1643	FTL_1792
<i>dsbB</i> , disulfide bond formation protein [EC:1.8.5.9] (K03611) (not mentioned in Figure)	FTT_0107c	FTN_1608	FTL_1670
Peroxidase:			
Catalase/oxidase, <i>katG</i> ; [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* 25,017.

	CSH	GSH
Sample	$\mu\text{mol/g dry weight}$	
<i>Francisella novicida</i>	7.89 $\pm$ 0.51	3.00 $\pm$ 0.20
<i>Francisella philomiragia</i> 25,017	3.19 $\pm$ 0.12	3.05 $\pm$ 0.09

(Ireland et al., 2019).

Redoxin genes in *Francisella*

Other LMW thiols include glutaredoxins (Grxs) and thioredoxins (Trxs) (Arner and Holmgren, 2000), 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) (Russel and Holmgren, 1988), and protect against oxidative stress (May et al., 2019; Norambuena et al., 2018; Ma et al., 2019) 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 (Fig. 1F). Gor is present in *Francisella* cytoplasm but was not essential for virulence either *in vivo* or *in vitro* (Table 1, Fig. 1D) (Alkhuder et al., 2009; Ramsey et al., 2020). There are 3 *Francisella* genes coding for glutaredoxins (Fig. 1F) (Russel and Holmgren, 1988; Cheng et al., 2017) (Table 1). In *Francisella*, expression of glutaredoxin 1 (*grxA*) gene is upregulated upon intramacrophage growth (Wehrly et al., 2009). GrxA enzyme is able to regenerate the reduced form of GSH following an oxidizing event (Masip et al., 2006). 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* (Ireland et al., 2019). Similarly, Asare et al. showed a 2-log reduction for *grxA* mutants in human macrophage U937 cell line (Asare et al., 2011; Asare and Abu Kwaik, 2011) using the *F. novicida* transposon insertion mutant library (Gallagher et al., 2007). (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 (Asare and Abu Kwaik, 2011).) On the other hand, in several other virulence screens in *Francisella*, no *grx* mutants were identified as having defects in intracellular replication or virulence (Weiss et al., 2007; Meibom and Charbit, 2010). 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 (Weiss et al., 2007). 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 (Read et al., 2008). 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 (Cheng et al., 2017; Carmel-Harel and Storz, 2000; Lu and Holmgren, 2014). These enzymes are encoded by the genes *trxA1*, *trxA2* and *trxB* respectively in *Francisella* (Table 3, Fig. 1E) (Rohmer et al., 2007). Deletion of thioredoxin reductase gene *trxB* (*trxR*) showed very little impact in the *Francisella* oxidative stress response (Ma et al., 2016). A 2.5Å crystal structure of the *Francisella* *trxB* protein has been deposited in the Protein Database (PDB 6BWT). Knockouts of the thioredoxin *trxA1* gene have shown significant impacts on the virulence of different pathogenic gram-negative bacteria (May et al., 2019a, 2019b, Carmel-Harel and Storz, 2000). *trxA1* was identified as being critical in *Francisella* virulence studies (Kraemer et al., 2009; Ma et al., 2022). 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 h 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 (Kraemer et al., 2009). 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 (Kraemer et al., 2009). Despite their weakened ability to infect, *trxA1* mutants were still able to spread to the spleen and liver following inhalation (Kraemer et al., 2009). Another study reported a 3-fold reduction in growth inside U937 macrophages for *F. novicida* *trxA1* mutants (Asare and Abu Kwaik, 2011). 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 (Ma et al., 2022). 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 (Ma et al., 2022; Ma et al., 2019). Interestingly, it has been proposed that the TrxR-Trx systems may be a target for antibiotic development in some bacteria (Lu and Holmgren, 2014).

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 (Murray and Wynn, 2011) 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 (Guina et al., 2007; Ramond et al., 2014; Ma et al., 2016, 2019, 2022; Honn et al., 2017, 2012; Lenco et al., 2005; Marghani et al., 2021). 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 (Lindgren et al., 2007).

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) (Alharbi et al., 2019). 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 (Alharbi et al., 2019). 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) (Binesse et al., 2015; Ireland et al., 2019; Alharbi et al., 2019), while Ireland et al. found it to be an essential gene in *F. tularensis* Schu S4 (Ireland et al., 2019). In addition, *ahpC* is required for virulence in mice in *Francisella* LVS infections (Alharbi et al., 2019), but is not essential in *F. novicida* for *in vitro* growth (Gallagher et al., 2007).

While most Gram-negative bacteria have an alkyl hydroperoxide reductase enzyme, AhpC, that requires AhpD or AhpF proteins as reductants to recycle the AhpC (Poole, 2005), *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* (Alharbi et al., 2019; Djuiika et al., 2013) (Fig. 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 (Fig. 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 (Alharbi et al., 2019).

Glutathione protects against oxidative stress

GSH can donate electrons to oxidants (Reniere, 2018) or it can participate in electron relays involving a GSH peroxidase, peroxiredoxins, and thiol transferases where they donate electrons for the reduction of peroxides (Binesse et al., 2015; Fourquet et al., 2008). In the process, GSH is oxidized, where two GSH molecules form a disulfide bond to produce GSSG (oxidized GSH), which is reduced by Gor (Fig. 1D) (Masip et al., 2006). In addition, GSH can protect exposed CSHs in proteins via S-glutathionylation (Maulik et al., 2009). S-glutathionylation of an exposed CSH residue has been shown to be catalyzed by Grx's (Tung et al., 2018). The mixed disulfide bonds are reduced by Trxs and Grxs in a process called deglutathionylation (Arner and Holmgren, 2000) (Fig. 1F) once the source of the oxidative stress is removed (Maulik et al., 2009).

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 (Fig. 1C). In *F. novicida*, the GSNOR *adhC* gene is FTN_0409. This protein was detected in *F. novicida* and *F. philomiragia* Outer Membrane Vesicles (Pierson et al., 2011). 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

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. \(2017\)](#). 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 (Lindgren et al., 2007).	<i>F. novicida</i> (Gallagher et al., 2007); Schu S4 (Larsson et al., 2005); LVS (Barabote et al., 2009).	From Holland et al. (LVS and Schu S4) (Holland et al., 2017).	From Murch et al. (Schu S4) (Murch et al., 2017).	From Bent et al. (LVS) (Bent et al., 2013).	Ramsey et al. (2020) LVS unless otherwise indicated. (Ramsey et al., 2020)		From Ireland et al. (Schu S4) (Ireland et al., 2019).
Gene ID	Gene Function	Locus number (<i>novicida</i>); (SchuS4); (LVS)	Normalized Fold Change BHI/MHB	Change Under Stringent Response	4 h/8 h infection	Essential in Macrophage Infection	Essential for <i>in vitro</i> growth	Essential <i>in vivo</i> (rat)
<i>oxyR</i>	Regulator of Oxidative Stress Response	FTN_0959; FTT_0556; FTL_1014.	1.5	No data	No Change at 4 h/ Up Regulated at 8 h	Not essential (Ramsey et al., 2020)	Not essential	No effect reported (Ireland et al., 2019)
<i>mglA</i> #/\$	Master Regulator of FPI Genes	FTN_1290; FTT_1275; FTL_1185.	0.6	No data	No Change at 4/8 h	Not essential (Ramsey et al., 2020) Essential in Fn replication in U937 cells (Asare and Abu Kwaik, 2011)	Not essential LVS (Ramsey et al., 2020 ; Su et al., 2007) Essential SchuS 4 (Ireland et al., 2019)	No effect reported (Ireland et al., 2019)
<i>gshA</i> #	Catalyzes first step of GSH synthesis	FTN_0277 (No mutants recovered); FTT_0367c; FTL_1304.	2.0	No data	No Change at 4/8 h	Not essential (Ramsey et al., 2020)	Not essential (Ramsey et al., 2020 ; Su et al., 2007) Essential Schu S4 (Ramsey et al., 2020 ; Ireland et al., 2019)	No effect reported (Ireland et al., 2019)
<i>gshB</i> #	Catalyzes second step of GSH synthesis	FTN_0804 (No mutants recovered); FTT_0926; FTL_1284.	1.7	No data	Down regulated at 4 h/ No Change at 8 h	Not essential LVS (Ramsey et al., 2020)	Not essential LVS (Ramsey et al., 2020 ; Su et al., 2007) Essential Schu S4 (Ramsey et al., 2020 ; Ireland et al., 2019)	No effect reported (Ireland et al., 2019)
<i>gshpx/ gpx</i>	Glutathione peroxidase	FTN_0698 Not essential; FTT_0733; FTL_1284.	0.8	No data	No Change at 4/8 h	Not essential (Ramsey et al., 2020)	Not essential (Ramsey et al., 2020)	No effect reported (Ireland et al., 2019)
<i>Peroxidase</i>	Dyp-type peroxidase family protein	FTN_1624; FTT_0086; FTL_1773.				Not essential (Ramsey et al., 2020), Essential in Fn replication in U937 and S2 cells (Asare and Abu Kwaik, 2011)	Not essential.	No effect reported (Ireland et al., 2019)
<i>adhC</i>	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 (Ireland et al., 2019)	Not found	Not present
<i>ahpC</i> \$/ **/ #	Glutathione-dependent peroxidoredoxin	FTN_0958 Not essential; FTT_0557; FTL_1015.	1.7	No data	Down regulated at 4 h/ No Change at 8 h.	Not essential LVS in Mø (Ramsey et al., 2020 ; Alharbi et al., 2019), Essential SchuS4 (Binesse et al., 2015 ; Ireland et al., 2019 ; Alharbi et al., 2019), Essential in Fn replication in U937 and S2 cells ()	Not essential LVS (Ramsey et al., 2020) Essential Schu S4 (Ireland et al., 2019)	Essential murine LVS infection (Alharbi et al., 2019) No effect reported for Schu S4 in rat (Ireland et al., 2019)

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Table 3 (continued)

Type of Data			Proteomics	RNA-Seq	Transcriptomics	Tn-Seq		TraDIS
Source & Strain	Francisella LVS and Schu S4 (Lindgren et al., 2007).	<i>F. novicida</i> (Gallagher et al., 2007); Schu S4 (Larsson et al., 2005); LVS (Barabote et al., 2009).	From Holland et al. (LVS and Schu S4) (Holland et al., 2017).	From Murch et al. (Schu S4) (Murch et al., 2017).	From Bent et al. (LVS) (Bent et al., 2013).	Ramsey et al. (2020) LVS unless otherwise indicated.(Ramsey et al., 2020)		From Ireland et al. (Schu S4) (Ireland et al., 2019).
Gene ID	Gene Function	Locus number (<i>novicida</i>); (SchuS4); (LVS)	Normalized Fold Change BHI/MHB	Change Under Stringent Response	4 h/8 h infection	Essential in Macrophage Infection	Essential for <i>in vitro</i> growth	Essential <i>in vivo</i> (rat)
<i>ahpC</i> /TSA		FTN_0973 (Meireles Dde et al., 2014) Not essential; Not found in Schu S4 or LVS.	Not present	Not present	No data	Asare and Abu Kwaik, 2011) Not present	Not present	Not present
<i>Gor</i>	Glutathione reductase	FTN_0835 Not essential; FTT_0955c; FTL_1248.	1.1	No data	Down regulated at 4 h/ No Change at 8 h	Not essential (Ramsey et al., 2020)	Not essential (Ramsey et al., 2020)	No effect reported (Ireland et al., 2019)
<i>ggt</i> **/\$	Gamma-glutamyl trans-peptidase	FTN_1159 Not essential; FTT_1181c; FTL_0766 * Intracellular growth defect.	0.9	No data	Up Regulated at 4 h / No Change at 8 h	Essential (Alkhuder et al., 2009; Ramsey et al., 2020) (−4.7 log2FC)	Not essential (Ramsey et al., 2020)	Essential for rat infection (Ireland et al., 2019). Also macrophages, mouse, hepatocytes.
<i>cydD</i> / <i>cydC</i>	CSH/ GSH ABC transporter permease/ ATP-binding protein	FTN_0642/ FTL_0641; FTT_1335/ FTT_1336; FTL_1495/ FTL_1496.	1.6	No data	Down regulated at 4 h (0.38) and upregulated at 8 h (1.35)	Not essential (Ramsey et al., 2020)	Not essential <i>F. novicida</i> (Gallagher et al., 2007), & Schu S4 (Ireland et al., 2019) <i>in vitro</i>	No effect reported (Ireland et al., 2019)
<i>chaC</i> \$	γ-glutamyl-cyclotransferase (GGCT) enzyme	FTN_0599; FTT_0509c; FTL_1548.	0.6	No data	No Change at 4/8 h.	Essential (Ramsey et al., 2020) (−8.8 log2FC)	Not essential Schu S4	No effect reported (Ireland et al., 2019)
<i>dptA</i> **/\$	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 h (3.81) and at 8 h (2.41). Also upregulated at 2, 4, 8, 12, 16, 24 h (Wehrly et al., 2009)	Essential (Ramsey et al., 2020) (−4.94 log2FC)	Not essential LVS (Ramsey et al., 2020)	Essential for rat infection (Ireland et al., 2019). Also macrophages, and Epithelial cells.
<i>gadC</i> #/\$	glutamate: γ-aminobutyrate (GABA) exchanger	FTN_0571 FTT_0480c FTL_1583	No data	No data	Up Regulated at 4 h (1.6) but Down at 8 h (0.73).	Not essential (Ramsey et al., 2020), Essential in Fn replication in U937 and S2 cells (Asare and Abu Kwaik, 2011)	Essential in LVS (Ramsey et al., 2020)	No effect reported (Ireland et al., 2019)
<i>grxA</i> #/\$	Glutaredoxin A	FTN_0982; FTT_0533c; FTL_0985	1.5	−1.82	No Change at 4/8 h.	Not essential (Ramsey et al., 2020), Essential in Fn replication in U937 cells (Asare and Abu Kwaik, 2011)	Not essential (Ramsey et al., 2020) Essential Schu S4 (Ireland et al., 2019)	Essential (Ramsey et al., 2020; Ireland et al., 2019)
<i>grxB</i>	Glutaredoxin B	FTN_1033; FTT_0650c; FTL_0923.	1.2	−1.65	No change at 4 h/ Up Regulated at 8 h.	Not essential (Ramsey et al., 2020)	Not essential (Ramsey et al., 2020)	No data
<i>grx3</i> /C#/ <i>reductase</i>	Glutaredoxin reductase	FTN_0983; FTT_0532c; FTL_0984.	1.6	No data	No Change at 4/8 h.	Not essential LVS (Ramsey et al., 2020)	Not essential (Ramsey et al., 2020) Essential Schu S4 (Ireland et al., 2019)	Essential (Ireland et al., 2019)
<i>glcA</i> \$	Lacto-glutathione lyase	FTN_1231; FTT_1212c; FTL_0732.	1.6	No data	Down Regulated at 4 h/ No change at 8 h.	Not essential (Ramsey et al., 2020), Essential in	Not essential (Ramsey et al., 2020)	No effect reported (Ireland et al., 2019)

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Table 3 (continued)

Type of Data			Proteomics	RNA-Seq	Transcriptomics	Tn-Seq		TraDIS
Source & Strain	Francisella LVS and Schu S4 (Lindgren et al., 2007).	<i>F. novicida</i> (Gallagher et al., 2007); Schu S4 (Larsson et al., 2005); LVS (Barabote et al., 2009).	From Holland et al. (LVS and Schu S4) (Holland et al., 2017).	From Murch et al. (Schu S4) (Murch et al., 2017).	From Bent et al. (LVS) (Bent et al., 2013).	Ramsey et al. (2020) LVS unless otherwise indicated.(Ramsey et al., 2020)		From Ireland et al. (Schu S4) (Ireland et al., 2019).
Gene ID	Gene Function	Locus number (<i>novicida</i>); (SchuS4); (LVS)	Normalized Fold Change BHI/MHB	Change Under Stringent Response	4 h/8 h infection	Essential in Macrophage Infection	Essential for <i>in vitro</i> growth	Essential <i>in vivo</i> (rat)
<i>pepA</i> **/\$	Amino peptidase A	FTN_0660; FTT_1318c; FTL_1479.	1.4	−2.51	No Change at 4/8 h.	Fn replication in U937 and S2 cells (Asare and Abu Kwaik, 2011) Essential (Ramsey et al., 2020) (−1.36 log2FC)	Not essential (Ramsey et al., 2020)	Essential (Ireland et al., 2019)
<i>pepB</i>	Amino peptidase B	FTN_0780; FTT_1094c; FTL_1108 (DA46–1685).	1.5	No data	No Change at 4/8 h.	Not essential (Ramsey et al., 2020)	Not essential (Ramsey et al., 2020)	No effect reported (Ireland et al., 2019)
<i>pepN</i>	Amino peptidase N	FTN_1798; FTT_1793c; FTL_1956.	2.4	−1.72	No Change at 4/8 h.	Not essential (Ramsey et al., 2020)	Not essential (Ramsey et al., 2020)	No effect reported (Ireland et al., 2019)
<i>trxA1</i> \$	Thioredoxin A1	FTN_1415; FTT_1445; FTL_0611.	1.1	No data	No Change at 4/8 h.	Essential (Ramsey et al., 2020; Kraemer et al., 2009; Ma et al., 2022) (−1.69 log2FC).	Not essential (Ramsey et al., 2020)	No effect reported (Ireland et al., 2019)
<i>trxA2</i>	Thioredoxin A2	FTN_0856; FTT_0976; FTL_1224.	No data	No data	Up regulated at 4 (2.83x) and 8 h (6.33x). **	Not essential (Ramsey et al., 2020)	Not essential (Ramsey et al., 2020)	No effect reported (Ireland et al., 2019)
<i>trxB/ trxR</i>	Thioredoxin reductase	FTN_0580; FTT_0489c; FTL_1571.	1.3	No data	No Change at 4/8 h.	Not essential (Ramsey et al., 2020)	Not essential (Ramsey et al., 2020)	No effect reported (Ireland et al., 2019)
<i>fupA</i> \$	GSH porin (Wang et al., 2023).	FTN_0444; FTT_0918; FTL_0439.	0.6	−2.05	Up Regulated at 4 h (1.97) but Down at 8 h (0.8).	Essential (Ramsey et al., 2020) (−1.55 log2FC). Essential in U937 cells (Asare and Abu Kwaik, 2011)	Essential in LVS (Ramsey et al., 2020)	No effect reported (Ireland et al., 2019)
<i>ngtA</i>	MFS superfamily (proposed GSH transporter) (Wang et al., 2023).	FTN_1011; FTT_0671; FTL_0946	No data	1.5	No data	Not essential (Ramsey et al., 2020),	Not essential (Ramsey et al., 2020)	No effect reported (Ireland et al., 2019)
<i>Cgc1</i> or <i>cgaA</i>	CobB/CobQ-like glutamine amidotransferase domain protein (proposed GSH breakdown) (Wang et al., 2023).	FTN_0435; FTT_0909; FTL_0429	No data	No data	Down Regulated at 4 hr (0.37) & 8 hr (0.36).	Not essential (Ramsey et al., 2020). Essential in Fn replication in S2 cells (Asare and Abu Kwaik, 2011)	Essential in LVS (Ramsey et al., 2020)	No effect reported (Ireland et al., 2019)

human pathogenic *F. tularensis* subsp. *tularensis* species such as Schu S4 or virulent *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 (Guina et al., 2007; Ma et al., 2016, 2022; Honn et al., 2012; Marghani et al., 2021). As in other Gram-negative bacteria, OxyR regulates the oxidative stress response in *Francisella* by regulating expression of over 125 genes (Ma et al., 2016). In *E. coli*, Grx and GSH can activate the OxyR oxidative stress regulator and begin the oxidative stress pathway (Carmel-Harel and Storz, 2000). In *Francisella* OxyR protein regulates the expression of classical antioxidant enzyme genes *ahpC* and *katG* (Ma et al., 2016; Lindgren et al., 2007). 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* (Ma et al., 2016; Spidlova et al., 2020). These proteins all play critical roles in oxidative stress in *Francisella* LVS and Schu S4 strains (Binesse et al., 2015; Lindgren et al., 2007; Alharbi et al., 2019; Kadzhaev et al., 2009). 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 (Ma et al., 2022). This contrasts with the *E. coli* model, in which OxyR enzyme is regulated by Grx and GSH (Aslund and Beckwith, 1999; Dubbs and Mongkolsuk, 2012; Hillion and Antelmann, 2015). The transcriptional master-regulator of *F. tularensis* virulence *mglA* (Wrench et al., 2013; Charity et al., 2009) controls the expression of many virulence genes in the *Francisella* pathogenicity island (Guina et al., 2007; Lauriano et al., 2004; Wrench et al., 2013; Bell et al., 2010). *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* (Guina et al., 2007).

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 (*mgIA* and *oxyR*). Whether these regulators can be directly or indirectly affected by GSH in *Francisella*, similar to that shown in *B. pseudomallei* (Wong et al., 2015) and *L. monocytogenes* (Hall et al., 2016) remains to be determined, with the regulation of *Francisella oxyR* by *trxA1* being an important recent advance in our understanding.

Glutathione 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* (Alkhuder et al., 2009; Ramsey et al., 2020) (Fig. 1A). GSH is at high levels in eukaryotic cell cytosols (estimated between 0.5 and 15 mM) (Deponate, 2013) and could be transported into the *Francisella* periplasm via porins such as FupA (Wang et al., 2023), potentially providing *Francisella* the chance to acquire much needed CSH for which it is auxotrophic (Meibom and Charbit, 2010) (Table 1). The gamma (γ) linkage between the CSH and glutamate residues is uniquely cleaved by Ggt enzyme (Table 1, Fig. 1A) to yield glutamate and Cys-Gly (L-cysteinyl-glycine) (Alkhuder et al., 2009; Masip et al., 2006). Cys-Gly is further processed to CSH and glycine by other peptidases (Alkhuder et al., 2009; Ku and Gan, 2019). The subcellular localization of *Francisella* Ggt enzyme is within the periplasm (membrane anchored) (Ramsey et al., 2020), which would facilitate its proposed activity against host derived GSH (Fig. 1A).

Alkhuder et al. 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” (Alkhuder et al., 2009; Gallagher et al., 2007; Meibom and Charbit, 2010). The studies demonstrated the impaired replication of the *Francisella* LVS *ggt* mutant (FTL_0766) in J774, RAW, and bone-derived macrophage cells (Alkhuder et al., 2009), and was confirmed by other labs (Maier et al., 2007). Other studies confirmed a role for *ggt* in *F. tularensis* Schu S4 replication in HepG2 cells (Qin and Mann, 2006). Furthermore, *ggt* mutants are less virulent in BALB/C mice (intraperitoneal route of infection) by three orders of magnitude (Alkhuder et al., 2009). Alkhuder et al. 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 (Alkhuder et al., 2009). 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 (Ramsey et al., 2020), 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 (Ireland et al., 2019). Interestingly, the optimal catalytic temperature for bacterial Ggt enzyme was determined to be between 37 and 60 °C (Castellano and Merlino, 2012). 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 (Fig. 1A) in a similar manner as proven in *E. coli* (Alkhuder et al., 2009; Ku and Gan, 2019). 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 Alkhuder et al. (2009).

DptA, the Cys-Gly transporter

DptA, the Cys-Gly dipeptide transporter, was identified in *F. tularensis* LVS (FTL_1251, FTT_0953c, FTT_0832) (Ramsey et al., 2020) (Fig. 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 (Ramsey et al., 2020) and infection in *F. tularensis* Schu S4 (Ireland et al., 2019). The peptide transporter *dptA* is not essential in LVS during *in vitro* growth but is required for intramacrophage growth (Ramsey et al., 2020; Ireland et al., 2019) and mutants strongly affect *F. tularensis* SchuS4 and replication in macrophages, epithelial cells and in the Fisher rat infection model (Ireland et al., 2019). 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 202.2 fold, the second most highly changed gene after uridylyl transferase in their study (90). Gene expression of *dptA* was increased during infection (Wehrly et al., 2009; Bent et al., 2013). In contrast, inactivation of *dptA* did not exert a high fitness cost for *F. novicida* (Wang et al., 2023).

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. (2020), 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 (Ramsey et al., 2020) (Fig. 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 (Ramsey et al., 2020). 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 (Ramsey et al., 2020). In the Fisher 344 rat model, *ggt*, *pepA*, and *chaC* were also found to be required for infection by *F. tularensis* SchuS4 (Ireland et al., 2019) (Table 3).

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

Recent studies by Wang et al. (2023) 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 (Asare and Abu Kwaik, 2011). Wang et al. (2023) 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. (2023) also newly identified a cytoplasmic glutamine

amindotransferase (GATase) enzyme, *cgcI/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 Drosophila S2 cells (Asare and Abu Kwaik, 2011). 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) (Fig. 1A), are present on *Francisella*'s cytoplasmic membrane (Ramond et al., 2014; Ramsey et al., 2020) to aid in "glutathione stealing". Both transporters move their cargo (the dipeptide or glutamate) from the bacterial periplasm to its cytoplasm (Ramsey et al., 2020). 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 to the inside of the *Francisella* via DptA to provide CSH and glycine for catabolism and growth. Alkhuder et al. (2009) and subsequently Ramsey et al. (2020) 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/cytosolic 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 (Ramond et al., 2014) 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 37°C. The recently demonstrated GSH-binding Ngta protein (Wang et al., 2023) 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 (Ramsey et al., 2020). 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 (Ireland et al., 2019). Notably, transposon mutants in *F. novicida* *gadC* showed a 4–5 log reduction in replication in U937 cells (Asare and Abu Kwaik, 2011) and *gadC* is also required for *in vitro* growth of *Francisella* LVS (Ramond et al., 2014; Ramsey et al., 2020).

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 a 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 (Wang et al., 2023). Bacterially produced GSH is proposed in this model to be exported to the periplasm via CydD, the proposed tripeptide exporter (Ramsey et al., 2020) (Fig. 1A). In *E. coli*, CSH-GSH ABC transporter CydD is a transporter which can export GSH from the cytoplasm to the periplasm (Pittman et al., 2005). 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. When the bacteria are infecting a mammalian host *in vivo*, GSH could be transported into the periplasm from the host via an outer membrane porin such as FupA (Wang et al., 2023) 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 growth *in vivo* as its deletion had no effect in the Fisher rat infection model (Ireland et al., 2019)(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* (Gallagher et al., 2007). These differences still require some additional study to understand if there are different pathways present in the different *Francisella* strains, such as suggested by the recent discovery of the role of *fupA/NgtA/cgaA* in *F. novicida* (Wang et al., 2023), and whether 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 (Ramond et al., 2014; Ramsey et al., 2020), 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 (Ramond et al., 2014; Ramsey et al., 2020; Ireland et al., 2019). 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 (Barel et al., 2015; Ramond et al., 2014). 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 of the host (Walker and van der Donk, 2016).

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 (Holland et al., 2017). We have summarized part of that dataset for the genes/proteins relevant to GSH in Table 3 along with some updates. Additionally, data from transposon sequencing (Tn-seq) and RNA-seq data were compiled from various 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

(Holland et al., 2017). Also of interest, the study by Murch et al. highlighted the downregulation of several GSH-related genes under stringent response including *gshB*, *GSH*, *grxA*, *grxB*, *pepB* and *trx* (Murch et al., 2017).

In a different approach, systems biology modeling was done 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 (Raghunathan et al., 2010) in contrast to the experimental results found when testing relevant mutants (Alkhuder et al., 2009). The systems biology modeling used may inadequately “understand” the metabolic pathways in *Francisella*. 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 versus 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 (Reniere et al., 2015) 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 (Wong et al., 2015). 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 (Brouwer et al., 2022). *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 (Vergauwen et al., 2010, 2003). The mechanisms for importing GSH vary between microorganisms.

While *Francisella* can synthesize GSH itself, it has many mechanisms to obtain it from exogenous sources which it appears to prefer to *de novo* synthesis when the bacteria is in a mammalian host. Recent studies have identified the outer membrane porin FupA as the import porin for intact GSH or Cys-Gly dipeptide and identified that NgfA protein plays a role for importing these peptides to the cytoplasm from the periplasm in *F. novicida*. Additionally, periplasmic GSH-cleaving enzymes, Ggt or ChaC, which are required for *Francisella*'s *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 bacterial cytoplasm via CydC/D proteins to modulate intracellular levels in the bacteria. 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:

- What are the potential functions of the multiple glutaredoxins in *Francisella*?
- There is a clear difference in the roles of *trx*A1 versus *trx*A2. Further elucidation of the mechanistic role of each of these thioredoxins would be helpful. Unlike in *E. coli*, *Francisella* *trx*A1 regulates the expression of *oxyR*, which then regulates the expression of the OxyR-dependent oxidative stress response (Ma et al., 2016, 2022; Spidlova et al., 2020). 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.
- The OxyR-regulated, GSH-dependent peroxiredoxin gene *ahpC* is protective against oxidative and nitrosative stress in *Francisella* sp. (Alharbi et al., 2019). 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 (Alharbi et al., 2019).

Credit author statement

MLVH and MR conceived the study. AM contributed to the Tables 1 and 3 and a preliminary draft of the manuscript. MLVH and MR edited and wrote the final version of the manuscript. All authors have approved the final version of this paper.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

This work was supported by the Independent Research Program at the National Science Foundation and NSF 1244611 to MR.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.crmicr.2023.100218.

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