Tn-Seq reveals hidden complexity in the utilization of host-derived glutathione in *Francisella tularensis*


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Abstract

Host-derived glutathione (GSH) is an essential source of cysteine for the intracellular pathogen *Francisella tularensis*. In a comprehensive transposon insertion sequencing screen, we identified several *F. tularensis* genes that play central and previously unappreciated roles in the utilization of GSH during the growth of the bacterium in macrophages. We show that one of these, a gene we named *dptA*, encodes a proton-dependent oligopeptide transporter that enables growth of the organism on the dipeptide Cys-Gly, a key breakdown product of GSH generated by the enzyme γ-glutamyltranspeptidase (GGT). Although GGT was thought to be the principal enzyme involved in GSH breakdown in *F. tularensis*, our screen identified a second enzyme, referred to as ChaC, that is also involved in the utilization of exogenous GSH. However, unlike GGT and DptA, we show that the importance of ChaC in supporting intramacrophage growth extends beyond cysteine acquisition. Taken together, our findings provide a compendium of *F. tularensis* genes required for intracellular growth and identify new players in the metabolism of GSH that could be attractive targets for therapeutic intervention.

Author summary

Prominent amongst the host-derived nutrients *Francisella tularensis* requires for intracellular growth is glutathione (GSH), a tripeptide which serves as an essential source of cysteine. Here we comprehensively identify those genes *F. tularensis* requires for intramacrophage growth and characterize two that play previously unrecognized roles in GSH utilization. One of these encodes a transporter of the dipeptide Cys-Gly which is a breakdown product of GSH, while the other encodes a member of the ChaC family of proteins which we show plays a role in GSH breakdown. Our findings uncover a critical role for a member of the proton-dependent oligopeptide transporter family in *Francisella* intramacrophage growth and provide the first example of a ChaC family enzyme acting to catabolize GSH in bacteria.

Introduction

*Francisella tularensis* is a facultative intracellular pathogen and the etiological agent of the disease tularemia, which can be fatal if left untreated [1]. Four subspecies of *F. tularensis* have been recognized (*tularensis*, *holarctica*, *mediasiatica* and *novicida*) which possess differential host ranges and cause disease with varying degrees of morbidity and mortality [2, 3]. While *F. tularensis* is capable of replicating in a variety of cell types, growth within macrophages is believed to be the primary mediator of disease [4, 5]. However, the precise virulence mechanisms employed for intracellular proliferation are largely uncharacterized.

*F. tularensis* initially enters cells through the endosomal pathway but must escape this degradative compartment to replicate within the cytoplasm. Perhaps the best characterized virulence factor utilized by *F. tularensis* is the type VI secretion system subtype 2 (T6SS2) pathway encoded by genes present on the so-called *Francisella* pathogenicity island (FPI) [6, 7]. This system has been demonstrated to secrete a set of effector proteins which mediate escape into the cytoplasm [8–10]. Additional factors known to be important for growth in host cells include many metabolic pathways, including those involved in purine biosynthesis and branched-
Tn-Seq reveals hidden complexity in the utilization of host-derived glutathione (GSH; γ-Glu-Cys-Gly). Francisella is a cysteine auxotroph and GSH is a necessary source of cysteine for intracellular replication [13]. This molecule is found at high concentrations (1–10 mM) in the cytoplasm of macrophages, as well as many other eukaryotic cells [14]. Existing literature suggests that the principal enzyme responsible for initiating degradation of GSH in Francisella is γ-glutamyl transpeptidase (GGT), which cleaves this tripeptide into glutamate and the dipeptide Cys-Gly [13, 15]. However, this is only the presumed first step of GSH utilization and the role of other factors that work in concert with GGT to facilitate GSH utilization has not been addressed. Moreover, Francisella encodes a member of the ChaC family of proteins which have been shown to contribute to the breakdown of GSH in eukaryotes [16–18]. Whether Francisella employs enzymes that act in parallel with GGT to breakdown GSH was not known.

While several screens have been employed to identify genes important for the intracellular replication of multiple F. tularensis subspecies [19–30], a comprehensive description of genes that are specifically required for the intramacrophage growth of this organism has been lacking. Here, we used transposon insertion sequencing (Tn-Seq) to identify the genes that are required for the intracellular growth of the live vaccine strain of F. tularensis (LVS). We leveraged the hits of our screen to define a unique pathway for the catabolism of GSH in Francisella. This pathway includes a promiscuous proton-dependent dipeptide transporter responsible for the import of Cys–Gly and a ChaC-family enzyme that can function alongside GGT to scavenge cysteine from GSH. Our work reveals unexpected complexity in the utilization of GSH by Francisella and provides a comprehensive catalog of genes required for the intracellular growth of this pathogen.

Results

Characterization of a highly saturated transposon insertion library for Tn-Seq

The ability of F. tularensis to cause disease is dependent principally upon its ability to grow within macrophages (reviewed in [3, 31, 32]). Although a number of genetic screens have been performed in several subspecies to identity genes required for this function [19, 21, 24, 25, 28], a consensus as to the bacterial requirements for intramacrophage growth remains lacking. To comprehensively identify Francisella genes critical for growth and survival within macrophages, as well as for growth in vitro, we created a saturated library of transposon insertion mutants in the live vaccine strain of F. tularensis (LVS) for use in transposon insertion sequencing (Tn-Seq) experiments. In order to use the insertion sequencing (INSeq) method of transposon insertion identification [33], we modified a previously used mariner transposon, which specifically inserts into TA dinucleotides [34]. Using the modified transposon encoding kanamycin resistance, we mutagenized LVS six independent times and combined approximately 800,000 kanamycin-resistant colonies into a single library.

We determined which genes are critical for in vitro growth by growing cells of the transposon mutant library on solid media and using extracted genomic DNA to specifically amplify bacterial transposon-chromosome junctions. Through this approach we identified 155,565 unique insertion events in our mutant library, corresponding to 74.6% of all the potential TA insertion sites (208,625) and one insertion event approximately every 12 bp (Fig 1A). To assess saturation of the mutant library at the individual gene level, we determined the percentage of TA sites in each gene with identified insertions. About 20% of genes had insertions in fewer than 10% of TA sites, suggesting these genes are essential under these growth conditions. In contrast, we could identify insertions in 90% or more of the TA sites for the majority of genes (73%), as would be expected for nonessential genes. The strong bimodal distribution of the percentage of TA sites disrupted per gene indicates that the library is highly saturated, as this distribution provides evidence that TA sites only lack an insertion when a transposon at that site would disrupt an essential function (S1B Fig).

In comparison, the prior most saturated transposon library used to screen for genes important for the intracellular replication of Francisella contained ~15,000 mutant clones (approximately one-tenth of the number of unique insertions in our mutant library) and use of the transposon site hybridization (TraSH) methodology precluded an assessment of the total number of unique insertions [24]. We used ARTIST, which determines essential genetic regions using a hidden Markov model [35], to identify 481 essential genes as well as 61 genes specifying proteins with both essential and non-essential domains (S1A Fig and S1 Table).

![Fig 1](https://journals.plos.org/plospathogens/article?id=10.1371/journal.ppat...)
Tn-Seq reveals hidden complexity in the utilization of host-derived glutathione (GSH) by Francisella tularensis. F. tularensis requires for growth and virulence [39]. The GGT enzyme mediates the first step in the breakdown of GSH, generating glutamate and the dipeptide Cys-Gly [40]. The F. tularensis enzyme contains a strongly predicted Sec signal sequence, suggesting that it localizes to the periplasm (S3 Fig). We therefore reasoned that utilization of Cys-Gly as a source of cysteine may require the action of a transporter that shuttles Cys-Gly from the periplasm to the bacterial cytoplasm where Cys-Gly could be broken down further to individual amino acid components. Moreover, we reasoned that the gene specifying a Cys-Gly peptide transporter would be amongst those we identified as essential for intramacrophage growth using Tn-Seq (Fig 1D, S2 Table).

Identification of a putative Cys-Gly transporter

A top hit from our in vivo Tn-Seq screen was FTL_1251, which encodes a putative proton-dependent oligopeptide transporter (POT) (Figs 1C and 2A and S2 Table). This group of transporters are part of the major facilitator superfamily and are characterized by the presence of 12–14 transmembrane domains, a signature motif of charged proton-binding residues within a transmembrane domain (ExxERF), and two PTR2 domains [41]. Each of these motifs was identified in FTL_1251 (residues 24–32, 72–96, and 153–167, respectively) and this protein contains 14 predicted transmembrane domains (Fig 2B). POT proteins often transport multiple peptides across the inner membrane, though these are generally related in sequence and chemical properties [42–44]. LVS is predicted to encode eight POT proteins; however, only FTL_1251, henceforth referred to as DptA, was hit in our screen (S2 Table). As no other putative peptide transporters were hit in our screen we hypothesized DptA contributes to intracellular growth of F. tularensis by acting within the GGT pathway for GSH catabolism.

Tn-Seq identifies genes and pathways critical for intramacrophage replication

In order to define LVS genes critical for intramacrophage growth, we infected J774A.1 murine macrophage-like cells with our transposon mutant library (with macrophages in ~80-fold excess of the complexity of our mutant library). After twenty-four hours, macrophages were lysed, and bacterial genomic DNA was extracted for library construction. Using Con-ARTIST [35] to compare a sample of the inoculum to the mutants that survived within macrophages, we identified 142 genes that were important for intramacrophage survival (Fig 1B and 1C and S2 Table).

Several lines of evidence indicate that our Tn-Seq-based screen for genes important for intramacrophage growth was unbiased and comprehensive. With regard to bias, if our infection conditions, isolation of bacterial genomic DNA from macrophages, or sequencing depth of recovered bacterial DNA generated a bottleneck, we would expect stochastic loss of transposon insertions in genes non-essential for intramacrophage growth. However, we did not identify a shift in insertion saturation in non-essential genes following growth of our mutant library in macrophages, strongly suggesting the lack of a bottleneck (S1B Fig). Instead, the TA insertion saturation remained strongly bimodal, consistent with recovery of essentially all mutants that were not defective for growth in macrophage. The comprehensive nature of our screen is highlighted by our identification of all thirteen genes encoding purine biosynthesis enzymes as either critical for survival in vitro or critical for growth or survival within macrophages (S2 Fig). Purine biosynthesis is critical for F. tularensis intracellular survival, yet prior screens had individually identified only up to eight genes in this pathway, as important for the process [19–20]. In addition, the gene encoding PigR, a transcription regulator critical for intramacrophage growth and virulence [34], was a strong hit in our screen despite its small size (336 bp) and previous lack of identification in other unbiased screens for factors important for intramacrophage growth or virulence (Fig 1D, S2 Table). Finally, our screen identified ~68% of the genes previously ascribed importance for growth in the J774 murine macrophage cell line [21, 25] while also revealing 89 additional genes as being critical for this process.

The ability of F. tularensis to grow within macrophages is dependent upon several nutrients that are derived from the host [36–39]. Prominent amongst these is GSH, a tripeptide whose catabolism provides a source of cysteine that F. tularensis requires for growth [13]. Consistent with previous findings, our screen identified ggt, which encodes a γ-glutamyl transpeptidase, as important for intramacrophage growth (Fig 1C and 1E and S2 Table) [13]. The GGT enzyme mediates the first step in the breakdown of GSH, generating glutamate and the dipeptide Cys-Gly [40]. The F. tularensis enzyme contains a strongly predicted Sec signal sequence, suggesting that it localizes to the periplasm (S3 Fig). We therefore reasoned that utilization of Cys-Gly as a source of cysteine may require the action of a transporter that shuttles Cys-Gly from the periplasm to the bacterial cytoplasm where Cys-Gly could be broken down further to individual amino acid components. Moreover, we reasoned that the gene specifying a Cys-Gly peptide transporter would be amongst those we identified as essential for intramacrophage growth using Tn-Seq (S2 Table).

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As a first step toward characterizing DptA, we probed its contribution to colonization of mice following infection by the aerosol route. Consistent with our Tn-Seq screen, disruption of \textit{dptA} resulted in significantly reduced proliferation within the lungs, as well as lowered dissemination to distal organs (Fig 2C and 2D). The observed \textit{in vivo} defects could be complemented by ectopic expression of \textit{dptA} from the Tn7 attachment site on the LVS chromosome. With the confirmation that the predicted transporter contributes to virulence, we set out to determine if DptA plays a role in GSH metabolism.

To evaluate the contribution of DptA to GSH metabolism, we measured the growth of a LVS \textit{dptA}(-) strain in Chamberlain’s defined media (CDM) containing a panel of cysteine-containing peptides. We also tested a strain of LVS in which we deleted the gene encoding GGT (LVS \textit{Δggt}), which was previously shown to be essential for the growth of LVS with GSH provided as a sole source of cysteine [13]. Indeed, we found that in LVS, GGT is required for growth on GSH and \textit{γGlu-Cys}, but not on cysteine or Cys-Gly (Fig 2E–2G and S4A and S4B Fig). Moreover, as expected, the \textit{in vitro} growth defects of the LVS \textit{Δggt} mutant strain in media supplemented with either GSH or \textit{γGlu-Cys} could be complemented by ectopic expression of \textit{ggt} from the Tn7 attachment site on the LVS chromosome (Fig 2E–2F). On the contrary, cells lacking DptA show no detectable growth on GSH or Cys-Gly and proliferate similarly to cells of the wild-type strain in the presence of \textit{γGlu-Cys} or cysteine (Fig 2E–2G and S4A and S4B Fig). Furthermore, the \textit{in vitro} growth defects of the LVS \textit{dptA} mutant strain in media supplemented with either GSH or Cys-Gly could be complemented by ectopic expression of \textit{dptA} from the Tn7 attachment site (Fig 2E and 2G and S4A and S4B Fig). These data suggest that DptA is required for the transport of Cys-Gly. Further, they suggest that under the conditions of our \textit{in vitro} growth assays, GGT and DptA constitute the single pathway for GSH degradation as well as transport of the resulting Cys-Gly breakdown product.

DptA is a promiscuous transporter that aids in cysteine acquisition during intramacrophage growth

To more definitively characterize the role of DptA in LVS, we turned to assays measuring the cellular uptake of radiolabeled GSH (\textit{H-glycine GSH} (\textit{H-GSH})). Cells of the LVS \textit{Δggt} mutant strain unable to harness GSH provided as a sole source of cysteine (Fig 3A) [13]. Disruption of \textit{dptA} likewise dramatically reduced intracellular radiolabel accumulation, supporting our hypothesis that DptA transports Cys-Gly, the product of GGT activity on GSH. Treatment of wild-type cells with CCCP, which dissipates the proton motive force, inhibited accumulation to the levels of the \textit{dptA} mutant strain, in-line with our assignment of DptA to the POT family (Fig 3B). Moreover, this result suggests that DptA plays a role in the transport of Cys-Gly. Further, they suggest that under the conditions of our \textit{in vitro} growth assays, GGT and DptA constitute the single pathway for GSH degradation as well as transport of the resulting Cys-Gly breakdown product.

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We next sought to determine the specificity of DptA. Competition for the uptake of $^3$H-GSH-derived species by various di- and tripeptides revealed that dipeptides with a C-terminal Gly are preferred substrates. Dipeptides with small, nonpolar C-terminal residues and a tripeptide composed entirely of Gly displayed detectable competition with $^3$H-GSH derivatives; however, their efficiency was significantly lower relative to Cys-Gly. Other tripeptides and dipeptides containing a bulky C-terminal residue were unable to compete with $^3$H-GSH products for uptake at the concentrations tested (Fig 3E). These results support our hypothesis that DptA is a key transporter of the Cys-Gly dipeptide in LVS.

Our *in vitro* results led us to a model in which GGT acts on GSH to generate Glu and Cys-Gly. These products are then transported into the cytoplasm via GadC and DptA, respectively, where they feed into general metabolism and to the production of cytosolic GSH (Fig 4A). If our model is correct, one would predict that the role of DptA during infection is cysteine acquisition, as has been shown for GGT \[13\]. Therefore, we tested the ability of a strain lacking DptA to grow in J774 macrophages with and without cysteine supplementation (Fig 4B). As expected based on our Tn-Seq findings, the *dptA* (-) strain displayed a significant intracellular growth defect without cysteine supplementation; however, addition of exogenous cysteine allowed this strain to proliferate to levels indistinguishable from the wild-type (Fig 4B). Taken together with our peptide uptake assays, these findings provide an explanation for our Tn-Seq data; DptA plays a critical role for LVS *in vivo* by transporting Cys-Gly, the breakdown product of host-derived GSH, which the cell requires for protein synthesis.
Identification of a second GSH-metabolizing enzyme, ChaC

In the course of our efforts to understand GSH metabolism in Francisella, we tested several mutants within *F. tularensis* subsp. *novicida* U112 in parallel with those generated in the LVS background. Surprisingly, we found that—in contrast to our results in LVS—GGT is dispensable for the growth of U112 in media containing GSH as a sole source of cysteine. The U112 Δggt strain failed to grow on γGlu-Cys as a sole cysteine source, indicating that the GGT protein of this species is synthesized and active (Fig 5A and 5B and S4C and S4D Fig).

Fig 5. Identification of a second GGT-independent pathway in *Francisella* for GSH catabolism mediated by ChaC.
(A-B) OD600 measurements of the indicated strains of U112 after 36 hrs of growth in CDM with GSH (A) and γGlu-Cys (B). Data are shown as the mean ± s.d. (C) GGT activity in the indicated strains of U112 and LVS determined via enzyme activity...
Tn-Seq reveals hidden complexity in the utilization of host-derived glutathione (GSH) by Francisella tularensis.

In eukaryotes, GGT-independent catabolism of cytosolic GSH occurs through the action of ChaC proteins, which cannot degrade GSH, one group of these enzymes, termed ChaC proteins, specifically catabolize GSH into 5-oxoproline and Cys-Gly. ChaC proteins are poorly characterized in prokaryotes, but their role in the metabolism of GSH is unclear. Nevertheless, ChaC may be the sole enzyme capable of hydrolyzing γ-glutamyl-containing peptides in these organisms. However, it is worth noting that the substrate used in this assay, L-γ-glutamyl-p-nitroanilide, is not a natural substrate of GGT and related enzymes. Thus, from these data, we could not rule out the possibility that other γ-glutamyl-targeting peptidases are present in LVS and U112. Nevertheless, these findings suggest that U112 possesses multiple pathways for accessing cysteine derived from extracellular GSH.

In eukaryotes, GGT-independent catabolism of cytosolic GSH occurs through the action of γ-glutamyl-cyclotransferase (GGCT) enzymes [45]. This family of proteins acts on a wide range of γ-glutamyl-conjugated peptides, and while the majority of GGCT proteins cannot degrade GSH, one group of these enzymes, specifically catabolizes GSH into 5-oxoproline and Cys-Gly [17, 46, 47]. ChaC proteins are poorly characterized in prokaryotes; the only such protein from bacteria that has been studied in detail is RipAY, a secreted virulence factor encoded by the plant pathogen Ralstonia solanacearum [48, 49]. Therefore, whether ChaC participates in GSH metabolism in bacteria in unclear. Nevertheless, we speculated that a second pathway for catabolism of extracellular GSH in U112 may involve the ChaC protein and searched its genome for sequences that could encode such an enzyme. These sequence searches, in conjunction with in silico structural analyses, defined FTN_0599 as the gene with the highest probability GGCT-family protein encoded by U112. Henceforth we refer to this protein as ChaC. Notably, ChaC contains an N-terminal transmembrane domain and a prior study demonstrated that the catalytic domain of its ortholog in LVS (98% amino acid identity)– one of the top hits in our Tn-Seq screen (Figs S5A and S5B)– resides in the periplasm and requires DsbA for proper folding [50]. We confirm that, like GGT, ChaC localizes to this compartment and is associated with cellular membranes (S5A and S5B Fig).

We hypothesized that U112, ChaC accounts for growth in GSH-containing media in the absence of GGT. To test this hypothesis, we generated an in-frame deletion of chaC in the wild-type and Δggt backgrounds of U112 and monitored growth on GSH. As expected, deletions of ggt or chaC alone had no effect on growth; however, a strain bearing mutations in both genes displayed markedly attenuated growth on GSH as the sole cysteine source. This phenotype was genetically complemented by expressing either gene ectopically from the chromosome (Fig 5A and 5B and S4C and S4D Fig). These findings suggest that in U112, ChaC can liberate GSH-derived species that support in vitro growth in media lacking free cysteine. Notably, this does not contradict our γ-glutamyl hydrolase activity assay, since ChaC enzymes are known to act at high specificity and are not predicted to tolerate the non-native substrate L-γ-glutamyl-p-nitroanilide. At this time, we lack an explanation for the differential contribution of ChaC to the in vitro growth of U112 and LVS on GSH. Western blot analyses ruled out expression as a simple explanation for our observations (S5C and S5D Fig).

While ChaC is required for the growth of LVS on GSH in vitro, as previously mentioned, ChaC was a top hit in our Tn-Seq screen performed in this strain (Figs 1C and 5D and S2 Table), suggesting this protein is important for growth in vivo and that its role is not entirely redundant with GGT. Intramacrophage growth assays confirmed the growth deficiency of LVS ΔchaC in this context. However, contrary to our results in the Δggt strain, exogenous cysteine did not complement the intramacrophage growth defect of ChaC (Fig 5E). Thus, the role of ChaC within macrophages appears not restricted to generating metabolizable cysteine from GSH. In total, these data highlight the existence of a previously unrecognized GGT-independent pathway capable of the catabolism of GSH by F. tularensis, but suggest the importance of ChaC in supporting intramacrophage growth extends beyond cysteine acquisition.

Discussion

Using Tn-Seq we have identified genes that are specifically required for the intramacrophage growth of F. tularensis. For many of the genes in this catalog the mechanistic basis for their role in intramacrophage growth is poorly understood. Utilization of host-derived GSH is essential for Francisella intramacrophage growth and we show that one of the genes from our compendium encodes a peptide transporter that plays a key role in transporting the GSH breakdown product Cys-Gly from the periplasm into the bacterial cytoplasm. In addition, we present evidence that another gene specifies a member of the ChaC family of proteins that is involved in the breakdown of exogenous GSH in Francisella.

Our Tn-Seq analyses were conducted with LVS, which is an attenuated derivative of a strain of F. tularensis subsp. holarctica, a type B strain. Recently a Tn-Seq study was performed to define genes essential for rat colonization of the highly virulent type A strain F. tularensis subsp. tularensis SCHU S4 [22]. In principle, this screen should define genes important for multiple aspects of infection, including bacterial uptake, immune evasion, and intracellular proliferation. Nevertheless, comparative analysis between the results obtained in the present study with those generated by Ireland et al demonstrate a high degree of overlap, suggesting that the genes important for infection in the host are involved in intramacrophage growth. In relation to genes important for the utilization of host-derived GSH, ggt, dptA and chaC were found to play critical roles during infection of the rat with SCHU S4 as assessed by Tn-Seq [22]. Thus, the proteins that play central roles in the metabolism of GSH, which are highly conserved amongst different species of F. tularensis, are important for the intramacrophage growth of LVS as well as for the fitness of SCHU S4 in the host. We note that GGT has previously been shown to be important for the virulence of both LVS and SCHU S4 in mice [13, 51], and that ChaC (i.e. FTL_1548) has been shown to be important for the virulence of LVS in mice [50].

Many bacteria possess transporters for the uptake of intact exogenous GSH, thereby providing an alternative mechanism for the
Tn-Seq reveals hidden complexity in the utilization of host-derived glutathione (GSH). The results from our saturated Tn-Seq screen led to the identification and characterization of the Cys-Gly transporter, DptA. As a member of the POT family, DptA is powered by the PMF and is capable of transporting multiple di- and tri-peptides with varying affinities. Our results suggest that this protein constitutes the sole mechanism for the transport of Cys-Gly across the inner membrane, making DptA an attractive target for novel therapeutics (Fig 4A). Interestingly, DptA also represents the first member of the POT protein family to be implicated in bacterial pathogenesis.

For many years GGT was believed to be the sole enzymatic capable of catalyzing GSH; however, within the last decade, two additional proteins families have been implicated in the breakdown of this molecule. Members of one of these families, termed the Dug2p/3p complex, are restricted to fungi [56, 57] whereas members of the other, ChaC family proteins, belong to the y-glutamyl cyclotransferase family of proteins and degrade GSH into 5-oxoproline and Cys-Gly [17]. The majority of studies on ChaC proteins have focused on representatives from mice and humans; however, ChaC homologs can be found in both prokaryotes and yeast [16–19]. Multicellular organisms encode two unique copies of ChaC [16]. ChaC1, which participates in the initiation of apoptosis via the unfolded protein response, rapidly catalyzes GSH breakdown in vitro (Kcat = 225 ± 15 min⁻¹) [17, 18]. On the other hand, ChaC2 is constitutively expressed and displays much slower GSH turnover (Kcat = 15.9 ± 1 min⁻¹). These results led to the hypothesis that ChaC2 functions to catalyze GSH at a low basal rate, whereas ChaC1 functions in response to stress [16].

However, a recent study using an embryonic stem cell model proposed that ChaC2 maintains GSH homeostasis through competitive binding with ChaC1. The authors demonstrate that ChaC2 was capable of inhibiting ChaC1 degradation of GSH in a dose dependent manner, which was important for embryonic stem cell regeneration [58]. Whether this model for the role of ChaC2 is conserved in other cell types was not addressed. Prior to this work, the physiological function of bacterial ChaC proteins had not been investigated; however, in vitro studies of *Escherichia coli* ChaC revealed its catalytic properties mirror that of mammalian ChaC2 (Kcat = 13 ± 0.27 min⁻¹) [16].

Here we identify a ChaC homolog in *F. tularensis* and show this protein acts in a GGT-independent pathway to mediate GSH catabolism. To our knowledge, this is the first demonstration that a bacterial ChaC enzyme contributes to the metabolism of GSH. It is unclear why *F. tularensis* contains two distinct periplasmic enzymes for the catabolism of GSH. We established cysteine acquisition as the critical function of GGT in intramacrophage growth, yet the intramacrophage growth defect of cells lacking the GGT is unclear why.

Although this work clearly implicates ChaC in GSH metabolism, precisely how the protein benefits *Francisella* during infection remains to be defined. Our efforts to understand ChaC in *Francisella* may also shed light on the role of the protein in other bacteria. However, bacterial ChaC family proteins are diverse, not restricted to host-associated organisms, and most are not predicted to localize to the periplasm. This suggests that ChaC proteins may serve myriad roles in GSH metabolism idiosyncratic to the lifestyles and physiological needs of bacteria harboring it.

### Materials and methods

#### Ethics statement

Animal studies were conducted in compliance with the National Research Council Guide for the Care and Use of Laboratory Animals. All experimental procedures were specifically approved in advance by the University of Washington Institutional Animal Care and Use Committee, under protocol number 2671–06. Animals were group-housed according to experimental group in HEPA-filtered laminar flow cages with cage enrichment and unrestricted access to sterile food and water. The vivarium is managed by the University of Washington Department of Comparative Medicine in compliance with all policies and regulations of the Office of Laboratory Animal Welfare of the Public Health Service. The facility is fully accredited by the American Association for Laboratory Animal Care.

**Bacterial strains and growth conditions**

Except where otherwise noted, *F. tularensis* subsp. *holarctica* LVS and derivatives were grown aerobically at 37°C in either liquid Mueller-Hinton broth (Difco) supplemented with glucose (0.1%), ferric pyrophosphate (0.025%), and Isovitalex (2%) (MHB) or on cystine heart agar (Difco) supplemented with 1% hemoglobin (CHAH). *F. tularensis* subsp. *novicida* strains U112, MFN245 and derivatives were grown aerobically at 37°C in tryptic soy broth or agar supplemented with 0.1% (w/v) cysteine (TSBC or TSAC). *Escherichia coli* strains XL1-blue (Stratagene) or DH5α were used in vector construction. For selection, antibiotics were used at the
following concentrations: kanamycin 5 μg mL⁻¹ (LVS), 15 μg mL⁻¹ (U112) or 50 μg mL⁻¹ (E. coli), nourseothricin 5 μg mL⁻¹, hygromycin 200 μg mL⁻¹, and carbenicillin 150 μg mL⁻¹.

Transposon mutant library construction and growth in vitro

The mariner transposon delivery plasmid pSD26 [34] was modified to facilitate use of the INSeq protocol [60]. The intermediate plasmid pKL84 was created by amplifying the kanamycin resistance cassette (the F. tularensis LVS groES promoter driving the kanamycin resistance gene [aphA1]) from pSD26. The 5’ primer included DNA specifying EcoRV and MfeI sites and the 3’ primer included DNA specifying NcoI, Nhel, and EcoRV sites. The resulting fragment was digested with EcoRV. The mariner transposon delivery plasmid pSAM_Bt [33, 61], which contains Mmrl sites within the transposon inverted repeat sequences, was digested with MfeI and XbaI and treated with Klenow large fragment to fill in overhanging 5’ ends. The digested DNAs were ligated together to create the pKL84 plasmid, which contains an MfI site on the 5’ end of the kanamycin resistance cassette and NcoI, Nhel, and the non-unique XbaI sites on the 3’ end. To optimize transposase production for LVS, the promoter for rpoD of Bacteroides thetaiotaomicron was replaced by the 209 bp fragment of DNA upstream of the transposase in pSD26. The promoter-containing fragment was amplified from pSD26 using a 5’ primer that included DNA specifying a BamHI site and a 3’ primer that included DNA specifying an optimal RBS (AGGAGG) followed by a Ndel site and ligated into pKL84 digested with BamHI and Ndel, resulting in the final transposon delivery plasmid pKL91.

To construct the transposon mutant libraries, one microgram of transposon delivery plasmid pKL91 was used to transform F. tularensis LVS (essentially as described in [62]) in six independent electroporations and cells were plated on CHAH with 5 μg mL⁻¹ kanamycin. After two days, the resulting kanamycin-resistant colonies, approximately 800,000, were combined into a single library and frozen. For all Tn-Seq experiments, single aliquots of the library were removed, spread on CHAH with 5 μg mL⁻¹ kanamycin, and grown overnight as a confluent lawn. Separate aliquots were grown and used as the inocula for growth in MHB, kanamycin. After two days, the resulting kanamycin-resistant colonies, approximately 800,000, were combined into a single library and frozen. For all Tn-Seq experiments, single aliquots of the library were removed, spread on CHAH with 5 μg mL⁻¹ kanamycin, and grown overnight as a confluent lawn. Separate aliquots were grown and used as the inocula for growth in MHB, intramacrophage growth, or to assess mutants viable on solid media. To screen for mutants deficient in growth in liquid media, transposon mutant library cells were diluted to an OD of 0.005 in 50 mL MHB and grown for ~17 hours (~6.2 doublings) prior to gDNA extraction.

Transposon mutant library screen in macrophages

Approximately 1x10⁷ of the murine macrophage-like J774A.1 cells were plated on six 150 mm tissue culture plates (~6x10⁶ cells total) in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Gemini Bio-Products) (DMEM-F) and incubated overnight at 37°C with 5% CO₂. Macrophage were infected with the LVS transposon mutant library resuspended in DMEM-F at an MOI of approximately 1700. After 2 hours, cells were washed twice with PBS and covered with DMEM containing 10 μg mL⁻¹ gentamycin. Cells were incubated at 37°C with 5% CO₂ for 24 hours then washed twice with PBS and lysed with 1% saponin in PBS for 30 minutes. Lysates were combined and a small aliquot was removed for bacterial enumeration on CHAH. Bacterial cells were pelleted and genomic DNA was directly extracted from pellets (Epicentre MasterPure Complete DNA and RNA Purification Kit).

INSeq library construction and sequencing

Sequencing libraries were created from 5 samples: (1) input sample for mutants grown in liquid MHB, (2) mutants grown on solid media and not subjected to additional screening, (3) input sample for mutants grown in macrophage (4) output sample for mutants grown in liquid MHB, (5) output sample for mutants grown in macrophage. Libraries were generated from isolated gDNA as in [60], starting with linear PCR using 2 μg DNA per reaction. Libraries were pooled and sequenced using single-end 50 bp sequencing with a single read cluster kit using two lanes of an Illumina HiSeq2500 at 15 pM and 10 pM densities with 20% PhiX and no indexing read. Sequencing reads and processed data files have been deposited at GEO with the accession number GSE138658.

Tn-Seq data analysis

Sequencing libraries from distinct samples were demultiplexed and trimmed using cutadapt (version 1.16; [63]). Reads were mapped to the LVS genome (NC_007880) using bowtie (version 1.2; [64]) with a seed length of 16 and zero mismatches allowed. Custom scripts were used to identify reads corresponding to insertions in TA sites and tally only those insertions with reads mapping from both ends of the transposon. Datasets were normalized for positional bias and analyzed using EL-ARTIST [35] to identify genes essential for in vitro growth using the combined data from all samples grown on solid media. Hidden Markov model analysis resulted in gene classifications (“critical,” “non-essential,” and “both non-essential and critical domains”) after the following sliding window training parameters: 4 TA sites, P < 0.03. CON-ARTIST [35] was used to identify genes critical for growth in liquid media and in vivo by comparing inoculum samples to the samples recovered after growth in both MHB and macrophage, respectively. We considered those genes with insertions in at least 4 TA sites, an average fold change of two-fold or more, and with a p-value of less than or equal to 0.05 to be important for survival in a given condition.

Francisella mutant and complementation strain construction

The 3’ end of dptA is very close to an isftu2 repetitive element, making a deletion using allelic exchange difficult. Therefore, we disrupted this gene by plasmid integration. Briefly, the plasmid pEX_dptA-frag was created by modification of pKL02 [65], a suicide plasmid which confers resistance to kanamycin. A 441 bp fragment of the dptA gene, including DNA specifying a KpnI site, DNA corresponding to amino acids 51–197, a stop codon after amino acid 197, and DNA specifying an EcoRI site, was introduced into pKL02 that had been digested with KpnI and EcoRI. The plasmid pEX_dptA-frag was then electroporated into LVS cells and cells in which the plasmid pEX_dptA-frag integrated though a single homologous recombination event were selected on CHAH with 5 μg mL⁻¹ kanamycin.

In-frame deletion mutations in LVS and U112 were generated via allelic exchange. For LVS, the vector pEX18Kan was employed to make the in-frame deletions of ggt and chA C, as described previously [66]. To generate deletion vectors for allelic exchange in U112, 1000 bp of flanking regions of ggt and chA C were cloned into the BamHI and PstI sites of the vector pEX18-pheS-km [8]. U112 deletion strains were then generated as previously described [8]. For all mutants, strains containing the modified genetic
region of interest was confirmed by colony PCR and sequencing of PCR products generated from genomic DNA.

The mini-Tn7 system vector pMP749 was utilized for genetic complementation in both LVS and U112 [67]. For ggt and chaC from LVS, the open reading frame plus the intergenic regions upstream (including the native promoter region) and downstream of the genes were amplified by PCR and cloned into the HindIII and BamHI sites of pMP749. For dptA, pMP749 was modified using Gibson assembly to replace the kanamycin resistance cassette for one that encodes nourseothricin resistance. The open reading frame plus the intergenic regions upstream (including the native promoter region) and downstream of dptA were amplified by PCR and cloned into the HindIII and BamHI sites of pMP749- nourseothricin. Complementation strains were then generated using these vectors as described in [67]. Briefly, parental strains were initially transformed with the helper plasmid pMP720. Hygromycin-resistant colonies were then transformed with the appropriate pMP749-based vector. Kanamycin or nourseothricin-resistant colonies were then passage in the absence of hygromycin to cure the bacteria of pMP720. Integration downstream of the gmnS gene was confirmed by colony PCR. For the complementation of ggt and chaC in U112, the genes were cloned downstream of the constitutively active bfr promoter, and complemented strains were generated as previously described [68]. For localization of ChaC and GGT in U112, the genes encoding ggt and chaC were cloned downstream of the bfr promoter with either a N-terminal VSV-G epitope tag or phoA (from E. coli) gene fusions into the HindIII and BamHI sites of pMP749, and the strains were generated as described above for LVS or U112. As controls for protein localization, full length phoA and phoA lacking the associated Sec signaling sequence were cloned into the same sites of pMP749. For expression studies in U112 and LVS, the genes encoding ggt and chaC were cloned with their native promoter regions and a C-terminal VSV-G epitope tag into the HindIII and BamHI sites of pMP749, and the strains were generated as described above for LVS or U112.

Mouse infections

Female C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) and were 8–10 weeks of age when enrolled in experiments. Mice were exposed to aerosolized bacteria in a whole-body aerosol exposure chamber, as described previously [68, 69]. Briefly, stocks of each bacterial strain were grown to stationary phase at 37°C in supplemented Mueller-Hinton broth diluted in 20% glycerol, aliquoted, and stored at -80°C. For each experiment, aliquots of each strain of bacteria were thawed, streaked onto chocolate agar and incubated at 37°C for 24h. Colonies were harvested and suspended in PBS to a concentration of approximately 3 x 10^8 CFU/mL, as estimated by optical density and confirmed by quantitative culture. Cohorts of mice were exposed to aerosolized bacteria in a whole animal exposure chamber with a computer interface to control pressures and flows (Biaera Technologies, Hagerstown, MD). Bacterial aerosols were generated by mini-Heart nebulizers with a flow rate of 8 L/min at 40 psi. Dilution air was regulated at 11.5 L/min to maintain total chamber flow at 19.5 L/min during a 10-minute exposure. Actual bacterial deposition was determined by quantitative culture of the homogenized left lungs of four sentinel mice euthanized immediately after completion of the aerosol exposure. The remaining mice were returned to their cages. Mice were observed and weighed daily. At serial time points after infection, mice were euthanized with pentobarbital, exsanguinated by cardiac puncture, and left lungs were harvested and homogenized for quantitative culture on chocolate agar.

Growth curves

To assess bacterial growth on different cysteine sources, the indicated strains of Francisella were grown overnight in Chamberlain's defined media (CDM) [70] with 1 μM cysteine at 37°C with shaking. After ~16 hrs of growth, the cells were washed three times and resuspended in CDM lacking cysteine. The bacteria were cysteine starved for 2 hrs at 37°C with shaking to allow the cells to metabolize any remaining cysteine. The OD_600 of the cultures was then measured and the bacteria were diluted to an OD_600 = 0.1 in CDM with the indicated potential cysteine source at 100 μM. For U112, cultures were transferred in triplicate to a 96-well plate and the OD_600 of the cultures was monitored for 36 hrs in a plate reader. For LVS, 3 mL cultures inoculated as described above were grown at 37°C and the OD_600 was determined at the indicated time points.

GGT activity assays

GGT activity in the indicated strains of LVS and U112 was determined using the γ-Glutamyltransferase (GGT) Activity Colorimetric Assay Kit (Sigma Aldrich). To prepare bacteria for the assay, cells were grown overnight on CDM containing cysteine, then washed three times in CDM lacking cysteine. The OD_600 of the cells was determined and cultures were normalized to an OD_600 = 1 (~10^8 cells) in 1 mL CDM lacking cysteine. Cells were pelleted and resuspended in 100 μL of assay buffer provided in the kit. Cells were lysed by incubating at 50°C for 3 min and cellular debris was removed by centrifugation at max speed for 10 min. The assay was then conducted per the manufacturer's specifications. Activity was measured by reading the absorbance at 480 nM in a plate reader pre-warmed to 37°C.

GSH uptake assays

The indicated strains of LVS were grown for 16 hrs at 37°C in CDM supplemented with 1 μM cysteine. Cells were spun down, washed three times in uptake buffer (25 mM Tris pH 7.5, 150 mM NaCl, 5 mM glucose), then concentrated 20-fold. The OD_600 was measured and normalized to OD_600 = 10. Each reaction contained 20 μL cells, 5μL 100 μM GSH + 0.5 μCi 3H-GSH, and 25 μL uptake buffer or competitor substrate at the indicated concentrations (made in uptake buffer). Uptake was allowed to occur for 45 min at 37°C followed by quenching with 1 mL ice cold uptake buffer. Cells were pelleted and washed three times in 1 mL cold uptake buffer, followed by resuspension in 50 μL uptake buffer. Cells were then added to scintillation fluid and counts were measured over 1 minute.

Macrophage replication assays

Macrophage replication assays for LVS and derivative strains were performed as in [71]. Briefly, approximately 2 x 10^4 murine macrophage-like J774A.1 cells were infected with LVS or derivative strains at an MOI of 5–10. Culture media containing extracellular bacteria was removed after 2 hours of infection and replaced with DMEM-F containing 10 μg/mL gentamycin. After 2 or 24 hours of infection, macrophage were lysed for 30 minutes in 1% saponin in 1X PBS and plated for enumeration. For assays including cysteine, 5 mM cysteine was added when macrophage were seeded and this concentration was maintained throughout.
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except for the first bin which is 0%.

green), and the transposon insertion mutant library recovered after intramacrophage growth (light green). Each bin represents 10%

protein regions, or are non-essential

For expression studies, LVS and U112 strains expressing either GGT-VSV-G or ChaC-VSV-G were grown overnight on CDM agar plates. Cells were then back diluted into CDM lacking cysteine supplemented with 100 μM GSH at an OD600 = 0.1. Cells were grown to OD600 = 0.6–0.8 followed by normalization to OD600 = 0.5. Bacterial cell lysates were generated by re-suspending cells in buffer containing 500 mM NaCl2, 50 mM Tris pH 7.5 and 10% (v/v) glycerol followed by mixing 1:2 with SDS-PAGE sample loading buffer. The supernatant, corresponding to the soluble fraction, was concentrated 5-fold using a 5 kD cutoff, and was mixed 1:2 with SDS-PAGE sample loading buffer. All samples were then boiled at 95°C for 10 min and processed for Western blotting as described below.

For expression studies, LVS and U112 strains expressing either GGT-VSV-G or ChaC-VSV-G were grown overnight on CDM agar plates. Cells were then back diluted into CDM lacking cysteine supplemented with 100 μM GSH at an OD600 = 0.1. Cells were grown to OD600 = 0.6–0.8 followed by normalization to OD600 = 0.5. Bacterial cell lysates were generated by re-suspending cells in buffer containing 500 mM NaCl2, 50 mM Tris pH 7.5 and 10% (v/v) glycerol followed by mixing 1:2 with SDS-PAGE sample loading buffer.

For both expression and protein localization studies, extracted protein samples in SDS-PAGE sample buffer were boiled at 95°C for 10 min and loaded at equal volumes to resolve using SDS-PAGE. Proteins were then transferred onto nitrocellulose membranes which were subsequently blocked in TBST (10 mM Tris-base pH 7.6, 150 mM NaCl2 and 0.1% w/v Tween-20) with 3% (w/v) bovine serum albumin (BSA) for 30 min at 24°C. This was followed by incubation with a monoclonal α–VSV-G antibody (Sigma Aldrich diluted 1:5000), α–Tu44 antibody (BEI diluted 1:3000), α–OpiA antibody ([68] diluted 1:5000) or α–GroEL antibody (diluted 1:15000 provided by Karsten Hazlett, Albany Medical College, Albany, New York, United States) in blocking buffer for 2 hrs at 24°C. Following three washes in TBST, blots were incubated for 45 min in TBST with an α–Rabbit HRP-conjugated secondary antibody (Sigma Aldrich diluted 1:5000) or α–Mouse HRP-conjugated secondary antibody (Millipore diluted 1:5000). The blots were developed using Radiance HRP substrate (Azure Biosystems), visualized using the Azure Biosystems c600, and densitometry analysis was performed using Image J. Density of the proteins corresponding to GGT and ChaC were normalized to the density of the loading control protein GroEL for each sample. Samples were generated and analyzed by Western blot three independent times. Representative images were assembled using Adobe Illustrator CC 2015.

Supporting information

S1 Fig. Characterization of a highly saturated transposon library generated in LVS.

(A) HMM analysis using ARTIST [35] identified which of the 2020 annotated genes that are critical, encode critical and non-critical genes. These were subsequently blocked in TBST (10 mM Tris-base pH 7.6, 150 mM NaCl2 and 0.1% w/v Tween-20) with 3% (w/v) bovine serum albumin (BSA) for 30 min at 24°C. This was followed by incubation with a monoclonal α–VSV-G antibody (Sigma Aldrich diluted 1:5000), α–Tu44 antibody (BEI diluted 1:3000), α–OpiA antibody ([68] diluted 1:5000) or α–GroEL antibody (diluted 1:15000 provided by Karsten Hazlett, Albany Medical College, Albany, New York, United States) in blocking buffer for 2 hrs at 24°C. Following three washes in TBST, blots were incubated for 45 min in TBST with an α–Rabbit HRP-conjugated secondary antibody (Sigma Aldrich diluted 1:5000) or α–Mouse HRP-conjugated secondary antibody (Millipore diluted 1:5000). The blots were developed using Radiance HRP substrate (Azure Biosystems), visualized using the Azure Biosystems c600, and densitometry analysis was performed using Image J. Density of the proteins corresponding to GGT and ChaC were normalized to the density of the loading control protein GroEL for each sample. Samples were generated and analyzed by Western blot three independent times. Representative images were assembled using Adobe Illustrator CC 2015.

S2 Fig. The purine biosynthesis pathway is critical during macrophage infection by F. tularensis.

The purine biosynthesis pathway of F. tularensis [11] is depicted with the gene encoding the enzyme responsible for each step listed and colored according to the environment in which they were found to be essential (green, essential in vitro; red, dispensable in vitro but essential in macrophages).

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(PDF)
S3 Fig. GGT is predicted to localize to the periplasm in *F. tularensis*. Graphical representation of the GGT protein with predicted important features of the protein highlighted. Predictions were based on well-characterized homologs of GGT.

https://doi.org/10.1371/journal.ppat.1008566.s003 (PDF)

S4 Fig. Genes involved in GSH catabolism do not play a role in free cysteine utilization. (A-B) OD<sub>600</sub> measurements of the indicated strains of LVS after 16 hrs of growth in CDM without (A) or with (B) free cysteine added. (C-D) OD<sub>600</sub> measurements of the indicated strains of U112 after 36 hrs of growth in CDM without (C) or with (D) free cysteine added. Data in A-D are shown as the mean ± s.d.

https://doi.org/10.1371/journal.ppat.1008566.s004 (PDF)

S5 Fig. GGT and ChaC act within the periplasm and the proteins are expressed in both LVS and U112 under *in vitro* conditions. (A) PhoA-activity of U112 expressing the indicated protein or protein-fusion. Data are shown as the mean ± s.d. of the cumulative results of two biological replicates performed in triplicate. Asterisks represent statistically significant differences (Student's t test; ***p ≤ 0.0005) (B) Western blot analysis of the localization of ChaC in the indicated strains of U112. Tul4 and OpiA were used as controls for the membrane and soluble fractions respectively. (C) Western blot analysis of ChaC and GGT abundance in the indicated strains of U112 and LVS grown in CDM where GSH is the sole cysteine source. GroEL was utilized as a loading control. (D) Densitometry analysis of ChaC and GGT expression levels from triplicate Western blot analyses as that shown in (C). Data are shown as the mean ± s.d. of three biological replicates.

https://doi.org/10.1371/journal.ppat.1008566.s005 (PDF)

S1 Table. Classification of genes based on EL-ARTIST analysis of *F. tularensis* LVS Tn-Seq library mutants grown *in vitro*.

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S2 Table. Genes identified as important for intramacrophage growth of *F. tularensis* LVS using Con-ARTIST analysis.

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