Cysteine biosynthesis is a determinant of *Brucella ovis* stress survival and fitness in the intracellular niche

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Abstract

*Brucella ovis* is an ovine intracellular pathogen with tropism for the male genital tract. To establish and maintain infection, *B. ovis* must survive stressful conditions inside host cells, including low pH, nutrient limitation, and reactive oxygen species. These same conditions are often encountered in stationary phase cultures. Studies of stationary phase may thus inform understanding of *Brucella* infection biology, yet the genes that are important in *Brucella* stationary phase physiology remain poorly defined. We measured fitness of a barcoded pool of *B. ovis* Tn-himar mutants as a function of growth phase and identified *cysE* as a determinant of fitness in stationary phase. *CysE* catalyzes the first step in cysteine biosynthesis from serine. We provide genetic evidence that two related enzymes, CysK1 and CysK2, function redundantly to catalyze cysteine synthesis downstream of CysE. Deleting either *cysE* or both *cysK1* and *cysK2* leads to premature entry into stationary phase and reduced culture yield. These phenotypes are rescued by addition of cysteine or glutathione to the medium. We further show that deletion of *cysE* results in sensitivity to exogenous hydrogen peroxide. Finally, we demonstrate that *B. ovis ΔcysE* has no defect in host cell entry but is attenuated in macrophage-like cells and in ovine testis epithelial cells at one- and two-days post infection. Our study uncovered unexpected redundancy at the CysK step of cysteine biosynthesis in *B. ovis*, and demonstrated that cysteine anabolism is an important determinant of stationary phase entry in vitro and fitness in the intracellular niche.

Introduction

*Brucella* spp. are intracellular pathogens that have numerous mechanisms to contend with host-generated stressors and exploit host resources for growth. Within the host, brucellae are subject to nutrient limitation (1), phagosomal acidification (2), and direct attack from reactive oxygen and reactive nitrogen species (3) originating from the host-derived respiratory burst (4, 5). Dozens of genes involved in oxidative stress responses, acid stress responses, nutrient assimilation, and respiration have been implicated in the biology of *Brucella* infection (6). More recent studies have defined a role for the general stress response pathway in mitigation of multiple chemical stressors in vitro and in maintenance of chronic infection in vivo (7, 8). However, relatively little is known about the mechanisms *Brucella* spp. use to adapt to stresses encountered in stationary phase culture. The study of stationary phase has the potential to inform the discovery of genes that influence infection, intracellular replication, and survival (9) as there are postulated parallels between stationary phase physiology and the physiologic state of *Brucella* in the intracellular niche (1).

We sought to develop an approach to identify genes involved in stationary phase physiology in the ovine pathogen, *Brucella ovis*. *B. ovis* (10) is an understudied member of the *Brucella* clade that has a number of distinguishing genomic features (11). It is one of two naturally rough species among the classical *Brucella* group (12) and is the only species of this group that is non-zoonotic. The host environment inhabited by *B. ovis* is quite restricted: it is sexually transmitted and has a specific tropism for the male genital tract in rams (13–15). We previously constructed a randomly barcoded (RB) library of *B. ovis* Tn-himar mutants (16, 17), and in this present study we set out to develop this barcoded mutant library as a tool to identify *B. ovis* genes with fitness defects in stationary phase culture. We measured the relative fitness of RB Tn-himar mutants as a function of growth phase in a complex medium and discovered that disruption of the cysteine biosynthesis gene, *cysE*, resulted in the largest stationary phase fitness defect in
our experiment. Thus, our screen provides evidence for a link between a sulfur assimilation/cysteine biosynthesis pathway and stationary phase physiology. Recent studies have significantly advanced our understanding of the roles of carbon and nitrogen metabolism in Brucella physiology and infection (18, 19). In contrast, our knowledge of sulfur metabolism in Brucella spp. is relatively limited, though cysteine and methionine biosynthesis genes have been implicated in mouse infection in Brucella melitensis 16M (20). Following the results of our RB-TnSeq screen, we showed that a Brucella ovis strain harboring an in-frame deletion of cysE (ΔcysE) had diminished culture yield and thus entered stationary phase prematurely. We provide evidence that this phenotype results from defects in cysteine and glutathione (GSH) biosynthesis. B. ovis ΔcysE is sensitive to hydrogen peroxide treatment, and replication of B. ovis ΔcysE is attenuated in a THP-1 macrophage cell line and an ovine testis epithelial line. The B. ovis ΔcysE strain is thus disadvantaged with respect to metabolism and oxidative stress survival, and these defects likely contribute to reduced intracellular fitness. We further report genetic evidence that B. ovis encodes two cysteine synthase-family enzymes (CysK1 and CysK2) that each function downstream of cysE to synthesize cysteine in B. ovis, and likely other Brucella species.

Results

B. ovis cysE Tn-himar mutant strains have a fitness defect in stationary phase

We inoculated ∼1.5 × 10⁹ B. ovis RB Tn-himar strains into Brucella Broth in triplicate and collected samples at intervals throughout the growth curve: 0.05, 0.12, 0.9 and 2.4 OD₆₀₀ (corresponding to early logarithmic, logarithmic, late logarithmic, and stationary phase). Barcodes were PCR amplified, sequenced, and tallied as previously described (21) to assess the relative abundance of each mutant strain in each sample. Our analysis yielded composite fitness scores for 2638 of 3391 annotated genes in B. ovis (Data Set 1). Data for 118 mutants that exceeded a t-like test significance threshold ≥ 4 are presented in Fig. S1 (see Materials and Methods). We observed the largest relative fitness score changes at OD₆₀₀ = 2.4 (i.e., stationary phase) in this dataset.

To more rigorously assess mutants with fitness values that varied as a function of growth phase, we further filtered the genes to include only those with a fitness score ≥ |1| in at least one timepoint (see Materials and Methods). We hierarchically clustered the 64 genes that passed this cutoff (Fig. 1A, Data Set 1). We divided these clustered genes into four groups that displayed different fitness patterns throughout the growth curve (Fig. 1B). Mutations in group 1 genes resulted in no fitness defect during exponential growth,
but a fitness defect at OD_{600} = 2.4 (i.e. stationary phase). Genes in group 2 had negative fitness scores throughout the growth curve. These two groups contained the majority of mutants. Group 3 (four genes) had positive fitness scores in log phase and a negative fitness score in stationary phase, while group 4 (three genes) had null or positive fitness scores at all phases of the growth curve. We further clustered these genes by predicted functional category (Fig. S2A, Data Set 2). Genes encoding purine metabolism enzymes and tRNA modification enzymes were enriched in group 1, but the gene with the lowest fitness score in stationary phase, BOV_RS06060 (old locus tag BOV_1224), is annotated as a serine-O-acetyl transferase (cysE) (Fig. 1 and Fig. S2B). As such, we chose to further characterize the function of cysE in B. ovis.

B. ovis ΔcysE enters stationary phase prematurely and has reduced culture yield in vitro

B. ovis CysE has a high level of sequence identity (52%) and similarity (73%) with the well-characterized CysE enzymes of Escherichia coli and Salmonella enterica (22), and is clearly classified as CysE in the NCBI conserved domain database (E-value = 4.3e-122, https://www.ncbi.nlm.nih.gov/Structure/cdd). This protein is therefore predicted to execute the initial step in cysteine biosynthesis, specifically the addition of an acetyl group from acetyl-CoA to serine, producing O-acetylserine (Fig. 2A). To validate the cysE stationary phase phenotype observed in the RB TnSeq experiment (Fig. 1), we built a B. ovis strain harboring an in-frame deletion of cysE (ΔcysE). We grew ΔcysE in parallel with wild-type B. ovis ATCC 25840 (WT, Fig. 2B) and observed that ΔcysE enters stationary phase earlier and terminates growth at a lower density than WT, thus corroborating the TnSeq result. This phenotype was complemented by the addition of 4 mM cysteine to the medium (Fig. 2B) or by ectopic expression of the cysE gene from a lac promoter in the presence of IPTG (Fig. 2C). The ectopic overexpression of cysE in a WT B. ovis background did not modify growth kinetics or the growth curve shape compared to WT/pSRK-EV (Fig. 2C). We conclude that cysE and cysteine biosynthesis are necessary for normal B. ovis growth yield in Brucella Broth.

cysK1 and cysK2 function redundantly in cysteine biosynthesis

CysK catalyzes the biosynthetic step subsequent to CysE, namely the elimination reaction in which the acetyl group on O-acetyl-serine is displaced by sulfide to form cysteine (22) (Fig. 2A). Given the stationary phase phenotype of ΔcysE, and the fact that this defect was chemically complemented by cysteine, we expected that mutations in cysteine synthase (cysK) should phenocopy ΔcysE. However, strains with insertions in locus BOV_RS09280 (old locus tag BOV_1893), annotated cysK in the NCBI RefSeq database, had a wild-type phenotype

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**Figure 2. ΔcysE enters stationary phase prematurely; this growth defect is rescued by addition of cysteine to the growth medium**

**A)** Schematic of cysteine biosynthesis from serine. Teal arrows indicate cysteine biosynthesis enzymes annotated in the Brucella ovis genome (RefSeq accessions NC_009505 and NC_009504). Enzymes: CysE (BOV_RS06060, cysE, O-acetylserine transferase); CysK1 (BOV_RS09280, cysK, cysteine synthase A). **B)** Representative growth curves of wild type (WT, circles), and ΔcysE (triangles) with (gray and ochre, respectively) or without (black and teal, respectively) addition of 4 mM cysteine (Cys) to the growth medium. **C)** Representative growth curves of WT carrying the pSRK empty vector (EV, black circle) or pSRK-cysE (gray square), and ΔcysE carrying pSRK (teal triangle) or pSRK-cysE (purple diamond) in BB with 1 mM IPTG, 50 μg/ml Kan. Error bars represent standard deviation of technical replicates in representative experiments.
Bacteria play a central role in the mitigation of a variety of environmental stressors such as reactive oxygen species (ROS). Given that the Brucella species are intracellular pathogens, it is important to establish whether the bacterium is equipped to handle oxidative stress.

We hypothesized that defects in cysteine biosynthesis would have consequences on GSH synthesis and sensitize cells to oxidative stress. We thus attempted to complement the ΔcysE growth phenotype by adding GSH to the medium (Fig. 4B). GSH supplementation partially complemented the ΔcysE growth yield defect. GSH limitation may directly contribute to premature entry of B. ovis ΔcysE into stationary phase, or GSH addition may restore cysteine homeostasis upon GSH catabolism. Since GSH is known to be involved in decomposition of hydrogen peroxide to water (23) (Fig. 4A), we assessed whether ΔcysE was more sensitive to 

\[ H_2O_2 \] stress. We grew WT and ΔcysE to stationary phase, washed the cells, treated them with 

\[ H_2O_2 \] for one hour in phosphate buffered saline solution, and then enumerated CFU. The ΔcysE strain was ~2000-fold more sensitive to 

\[ H_2O_2 \] than WT, and this sensitivity was rescued by either the addition of cysteine or glutathione to the medium during growth (Fig. 4C). The hydrogen peroxide sensitivity phenotype of ΔcysE was genetically complemented by expression of cysE from the lac promoter of the pSRK plasmid (ΔcysE/pSRK-cysE) (Fig. 4D). These results show that B. ovis ΔcysE has an increased sensitivity to exogenous 

\[ H_2O_2 \].

**Intracellular fitness, but not host cell entry, is reduced in ΔcysE**

*Brucella* spp. primarily reside inside mammalian host cells. There are many challenges to cell survival and growth in the intracellular niche including nutrient limitation and exposure to stressors such as reactive oxygen species (ROS) (6). Considering the growth and hydrogen peroxide sensitivity phenotypes of ΔcysE, we...
decided to test whether fitness of the ΔcysE strain is compromised in the intracellular niche. We infected a human monocytic cell line, THP-1, that we differentiated into macrophage-like cells. Although entry (2 hrs post-infection, p.i.) to the macrophage was unaffected by the lack of cysE, there was a significant loss in recoverable colony forming units (CFU) 24 hrs p.i. relative to WT (Fig. 5A). Thus B. ovis ΔcysE enters the macrophage like WT but, once inside, survival and/or replication are hindered. This intracellular infection defect was partially complemented by expression of cysE from a plasmid (Fig. 5B). We attribute partial complementation to the fact that the cysE was expressed from an IPTG-inducible promoter on a replicating plasmid; there are challenges with full induction of genes from heterologous promoters in an intracellular infection context.

Given the ability of Brucella to infect multiple mammalian cell types, we next tested whether the in vitro infection phenotype of ΔcysE was particular to macrophages. Specifically, we infected a sheep testis epithelial cell line (OA3.ts) (24), which is derived from a relevant B. ovis host tissue type. Again, entry was unaffected by the lack of cysE (Fig. 5C). Recovered CFU were significantly lower at 24 hr post-infection for ΔcysE compared to WT, about 10-fold loss. This phenotype was partially complemented by ectopic expression of cysE.

![Diagram](https://via.placeholder.com/150)

**Figure 4.** *B. ovis* ΔcysE is sensitive to H₂O₂ treatment; ΔcysE growth defect and peroxide sensitivity is mitigated by glutathione. 
A) Schematic of glutathione metabolism. Enzymes annotated in the *Brucella ovis* genome (RefSeq accessions NC_009505 and NC_009504) are indicated in bold: GshA (BOV_RS13935, gshA, glutamate—cysteine ligase), GshB (BOV_RS10075, gshB, glutathione synthase), and Gor (BOV_RS04850, gor, glutathione disulfide—reductase). GSH (glutathione, reduced state); GSSG (glutathione disulfide, oxidized state). B) Growth of wild type (circles) or ΔcysE (triangles) in BB with (gray and ochre, respectively) or without (black and teal, respectively) 4 mM GSH added to the medium. Error bars represent standard deviations and may be smaller than symbol size. C) Hydrogen peroxide survival assay showing the log₂ ratio of CFU of treated (20 mM H₂O₂) versus untreated (mock treatment in PBS) cultures. Black bars represent wild type and teal bars represent ΔcysE strains. Addition of either 4 mM cysteine (+ Cys) or 4 mM GSH (+ GSH) to the medium is indicated by the shaded boxes. GSH was washed away from the culture prior to peroxide treatment. D) Same assay as in C but with plasmid bearing strains stressed with 15 mM H₂O₂. Black bars represent WT / pSRK, teal bars represent ΔcysE / pSRK-cysE, and purple bars represent ΔcysE / pSRK-cysE. p-values: * p < 0.05; ** p < 0.01; *** p < 0.001, **** p < 0.0001, calculated using one-way ANOVA (followed by Dunnett’s multiple comparison test, to ΔcysE in C or ΔcysE/ev in D). Error bars represent standard error of the mean for 3 or 4 independent experiments.
an understudied member of the mutants leads to cysteine metabolism A ovis these infection data provide evidence that infection and/or growth

| Hrs p.i. | Log(CFU/well) |
|----------|---------------|
| 0        | 3.0           |
| 24       | 3.5           |
| 48       | 4.0           |

*** p < 0.001, **** p < 0.0001. Infections were repeated 3 times using one ANOVA (Tukey’s multiple comparison test) significance at the 24 hr time point was assessed using one-way ANOVA (Tukey’s multiple comparison test). Asterisks indicate p-values comparing re-

from a plasmid (Fig. 5D). The magnitude of the ΔcysE defect 24 hrs p.i. was greater in THP-1 derived macrophages than in the epithelial OA3.ts cell line (about 64-fold vs 16-fold, respectively; Fig. 5S3). Altogether, these infection data provide evidence that an intact cysteine metabolism system is necessary for normal B. ovis survival and/or growth in intracellular niche of multiple mammalian cell types.

**Discussion**

A genome-scale search for B. ovis stationary phase mutants leads to cysteine metabolism

B. ovis is a widespread ovine pathogen that remains an understudied member of the Brucella genus. Using a RB TnSeq approach, we sought to identify genes that are important for B. ovis growth and/or survival in the late phase of axenic broth culture (i.e. stationary phase), with a larger goal of uncovering genes that are important for fitness in the intracellular environment. We identified multiple genes for which Tn-himar disruption resulted in reduced fitness in stationary phase (Fig. 1). Among the expected mutants in this dataset is rsh (Figs. S1, 1 and S2A, Group 2; Data Sets 1 and 2, BOV_RS03230), which controls the stringent response (25). Additionally, genes involved in purine metabolism (Figs. S1 and S2; Data Sets 1 and 2), including purF have reduced fitness in dense culture. In Mycobacterium smegmatis, PurF influences survival during stationary phase (26), and purine metabolism is known to be important for growth of multiple microbes in the intracellular and extracellular environments (27, 28). Multiple genes with a predicted role in tRNA modification also had diminished fitness in stationary phase. Transfer RNA modification enzymes have roles in translation quality control and can function to direct translation of specific transcripts under particular growth conditions (29). Given the phenotypes of tRNA modification mutants in stationary phase, it may be the case that these genes play a role of regulation of Brucella ovis physiology in the intracellular niche.

Tn-himar strains with insertions in cysE had the most diminished fitness in stationary phase, and cysE was therefore selected for follow-up studies. Sulfur and cysteine metabolism are central to microbial growth, and have been well studied in numerous pathogens (30). Based on the high level of sequence identity/similarity to well-studied CysE enzymes and established structural data on B. abortus CysE (31), B. ovis CysE is presumed to catalyze biosynthesis of O-acetylserylserine from acetyl-CoA and serine. The subsequent step in biosynthesis of cysteine from O-acetylserylserine requires displacement of the acetyl group by sulfide, a reaction that is catalyzed by CysK in many bacteria. Our growth data (Fig. 2B and C) clearly implicate cysE in the cysteine biosynthesis pathway, as the in vitro growth defect of ΔcysE is rescued by the addition of cysteine. These results support published data that Brucella spp. can assimilate cysteine as an exogenous organic sulfur source (16, 32).

Surprisingly, the growth phenotypes of strains with Tn-himar insertions in the gene annotated as cysK in the RefSeq database did not differ from wild type, which suggested redundancy at this biosynthetic step. Consistent with this hypothesis, we have presented genetic evidence that two related enzymes, CysK1 and CysK2, function redundantly to produce cysteine (Fig. 3). It is possible that CysK1 and CysK2 do not catalyze the same reaction, but rather determine cysteine biosynthesis through two distinct routes. A recent report
of such a case is the cystathionine β-synthase of *Helicobacter pylori*, which retains some O-acetylserine sulphydrylase activity (33); this enzyme shares some features of primary structure with *B. ovis* CysK2. CysK-family enzymes can also have functions beyond direct involvement in cysteine metabolism (34), which may influence interpretation of our results. Notably, *B. abortus* CysE (serine O-acetyltransferase) and CysK2 do not form a cysteine synthase complex (CSC) in vitro (35). This supports a model in which CysK2 participates in cysteine synthesis via a mechanism that differs from that catalyzed by the typical CysE-CysK CSC. We postulate that CysK1, rather than CysK2, binds to CysE to form the CSC in *Brucella*. The development of a defined medium that supports the growth of *B. ovis* would greatly facilitate future study of CysK1 and CysK2 functions in cells. Exploration of the possible intracellular fitness advantage gained by redundancy at the CysK step of cysteine biosynthesis is an interesting area of future investigation.

**Cysteine, glutathione and hydrogen peroxide stress**

The growth and peroxide survival defects of ΔcysE were partially rescued by addition of cysteine or glutathione to the medium. Though elevated intracellular cysteine enhances susceptibility to hydrogen peroxide stress in *Escherichia coli* (36, 37), we do not observe peroxide sensitization of WT or ΔcysE *B. ovis* upon addition of 4 mM cysteine. 4 mM cysteine was consistently more protective than 4 mM GSH in our assay. GSH is an important redox control molecule, but the protective effect of GSH supplementation against H₂O₂ may be indirect. Specifically, it’s possible that *B. ovis* transports and metabolizes some of the GSH to release cysteine, which is one of the three component amino acids of GSH. *B. ovis* is predicted to encode a γ-glutamylcyclotransferase (*BOV_RS09395*), which catalyzes the cleavage of GSH to form pyroglutamic acid and L-cysteinylglycine (38). The L-cysteinylglycine dipeptide could then be separated by peptidases to release cysteine. We nonetheless favor a model in which diminished GSH production (as a result of abolished cysteine production) in ΔcysE directly affects H₂O₂ detoxification and growth yield of ΔcysE (Fig. 4B-C). Glutathione metabolism is important in *B. ovis*: the gshA biosynthesis gene (Fig. 4A) is essential based on our published TnSeq dataset. Moreover, Tn-himar insertions in *BOV_RS04850* (old locus tag, *BOV_0978*), which is predicted to encode a glutathione-disulfide reductase (*gor*, Fig. 4A) – that reduces GSSG to GSH – resulted in a significant fitness disadvantage throughout the growth curve (Group 2, Figs. 1 and S2, Data Set 2).

**Cysteine and growth in the intracellular niche**

Our study further provides evidence that cysteine biosynthesis is necessary for normal *B. ovis* growth and/or survival inside mammalian host cells. Specifically, strains harboring deletions of cysE were not defective in host cell entry but had significantly reduced recoverable CFUs at 24 and 48 hrs post infection in mammalian macrophage-like and epithelial cell lines (Figs. 5 and S4). Nutritional restriction and enhanced sensitivity to oxidative stress (Figs. 2 and 4) likely both contribute to diminished fitness of *B. ovis* cysE mutants in these lines. Of note, the defect of the ΔcysE strain was more pronounced in the THP-1 macrophage-like line than in the testic epithelial line (Fig. S3). Sensitivity of ΔcysE to ROS (Figure 4) may underlie this difference in fitness between cell lines as macrophages typically have a more robust respiratory burst than epithelial cells (39).

Genome-scale *B. abortus* TnSeq studies by Sternon and colleagues (40) did not identify cysE as a gene that was important for infection of Raw 264.7 macrophages. We infected Raw 264.7 cells with *B. ovis* ΔcysE and observed attenuation after 24 hrs (Fig. S4), but the nature of the Sternon *et al*. experiment and our experiment differ in several ways. It is possible that the importance of cysteine metabolism in intracellular growth and/or survival may vary across *Brucella* species and between mammalian cell lines.

Cysteine and methionine metabolic pathways are interesting targets to combat various pathogens (30). An attractive characteristic of these pathways is that, in mammals, cysteine and methionine cannot be synthesized and must be acquired from diet. Thus, compounds that disrupt cysteine metabolism are not predicted to have direct negative effects on mammalian metabolism. In fact, O-acetylserine sulphydrylase (OASS; i.e. cysK) inhibitors are under investigation as therapeutics for *Mycobacterium tuberculosis* infections (41). Our work shows that genetic disruption of cysteine biosynthesis leads to a significant defect in the capacity of *B. ovis* to survive and/or replicate within host cells. Thus, this pathway may be a novel target for combating brucellosis.

**Materials and Methods**

**Bacterial strains and growth conditions**

*Brucella ovis* was grown on Tryptic Soy Agar (TSA, Difco Laboratories) plates, supplemented with 5% sheep blood (Quad Five) or in Brucella Broth (BB, Difco Laboratories, dissolved in tap water) for liquid cultures. Cells were incubated at 37 °C with 5% CO₂ supplementation. Kanamycin (Kan) 50 μg/ml, sucrose
(5% w/v) or isopropyl β-D-1-thiogalactopyranoside (IPTG, GoldBio) at 1 mM or 2 mM, were added when required.

*Escherichia coli* strains were grown in lysogeny broth (LB, Fisher Bioreagents) or on LB + 1.5% agar (Fisher Bioreagents) plates at 37 °C with Kan supplemented at a concentration of 50 µg/ml when required. *E. coli* WM3064 strain, used for conjugation, was grown in the presence of 300 µM diaminopimelic acid (DAP, Sigma-Aldrich), as it is a DAP auxotroph.

**Plasmid and strain construction**

*Deletion plasmid construction*

To build the deletion strains, fragments of approximately 500 bp upstream and downstream of target genes were amplified with KOD Xtreme Hot Start polymerase (Novagen). These fragments were built so that 9 bases at both the 5’ and 3’ ends of the gene were maintained, keeping the gene product in frame to minimize polar effects. Purified DNA from *Brucella ovis* ATCC 25840 was used as a template. Amplified fragments were gel purified (ThermoFisher Scientific) and assembled into the pNPTS138 suicide deletion vector (digested with HindIII and BamHI restriction enzymes, New England Biolabs) using Gibson assembly (New England Biolabs).

*Complementation plasmid construction*

To build plasmids for genetic complementation, *cysE*, *cysK1* or *cysK2* were PCR amplified from *B. ovis* ATCC 25840 with KOD Xtreme Hot Start polymerase, gel purified (ThermoFisher Scientific) and Gibson assembled into pSRK (42) that had been digested with Ndel and Kpnl restriction enzymes (New England Biolabs). *cysE, cysK1* or *cysK2* were cloned downstream of P<sub>lac</sub> (lactose, IPTG inducible promoter).

*Delivery of plasmids to B. ovis*

Constructed plasmids were transformed into chemically competent *E. coli* Top10 strains for plasmid maintenance. All plasmid inserts were confirmed by PCR and Sanger sequencing, and plasmids were delivered to *B. ovis* by conjugation using *E. coli* WM3064 as a donor strain. For conjugation, WM3064 donor strains were mated with *B. ovis* strains and spotted on TSA blood plates plus DAP and incubated overnight at 37 °C in a 5% CO<sub>2</sub> atmosphere. Mating spots were spread on TSA blood plates plus Kan (without DAP) to select for *B. ovis* plasmid acquisition. When deleting genes using the pNPTS138 plasmid, merodiploid clones were inoculated in Brucella Broth overnight to allow for a second crossover event, then spread on TSA blood plates plus sucrose (5% w/v) for counterselection. Single colonies harboring gene deletions were identified by patching clones on TSA blood plates with or without Kan. The putative deleted locus was PCR amplified using gene-flanking primers in Kan-sensitive clones, and the PCR fragment was resolved by gel electrophoresis to test whether the gene had been deleted. For a complete list of strains, plasmids, and primers, please see Data Set 3.

**Growth Curves**

Cells were inoculated from ~48hr-old TSA blood plates into BB at densities ranging from OD<sub>600</sub> 0.08 to OD<sub>600</sub> of 0.2. Growth was assessed spectrophotometrically measuring optical density at 600 nm (OD<sub>600</sub>). Growth curves were conducted at least three independent times with two or three technical replicated in each experiment. Representative curves are shown for each set of strains. Where indicated, cysteine (4 mM), GSH (4 mM), Kan (50 µg/µl), or IPTG (1 mM) were supplemented upon start of growth experiment to the liquid media.

**H<sub>2</sub>O<sub>2</sub> survival assays**

Cells were grown overnight in BB to stationary phase (OD<sub>600</sub> of ~2). Cells were pelleted and resuspended in Phosphate-Buffered Saline (PBS, Sigma) to achieve an OD<sub>600</sub> of 0.15. 200 µl of cells were added to 1.8 ml of PBS or PBS supplemented with fresh H<sub>2</sub>O<sub>2</sub> (15 or 20 mM final concentration), bringing the final OD<sub>600</sub> to 0.015. Cells were then incubated at 37 °C in 5% CO<sub>2</sub> for 1 hr before spotting aliquots of a 10-fold serial dilution series on TSA blood plates. CFUs were enumerated after 48 hrs incubation at 37 °C in 5% CO<sub>2</sub>. Experiments were repeated at least three times with each sample in duplicate or triplicate in each experiment.

**DNA extractions**

Cells from 1 ml of stationary phase culture were pelleted by centrifugation, washed once in PBS, and resuspended in 100 ul TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) supplemented with 1 µg/ml RNaseA. Cells were lysed by addition of 0.5 ml GES lysis solution (5 M guanidinium thiocyanate, 0.5 M EDTA pH 8.0, 0.5% v/v Sarkosyl) and 15 min incubation at 60 °C. 0.25 ml cold 7.5 M ammonium acetate (Fisher Bioreagents) was added, and mixture was incubated on ice for 10 min. 0.5 ml of chloroform (Fisher Bioreagents) was added to separate the DNA, samples were vortexed and centrifuged. Aqueous top phase was moved to a fresh 1.5 ml centrifuge tube and 0.54 volumes of cold isopropanol was added to precipitate the DNA. After centrifugation, isopropanol was discarded and pellets were washed.
Barcoded TnSeq

A *B. ovis* RB Tn-himar library was built and mapped as described (16). Briefly, *E. coli* APA752 (a WM3064 donor strain carrying a pKMW3 mariner transposon library) was conjugated into *B. ovis* bcaA1 (17) under atmospheric CO₂ conditions. Kan resistant colonies were collected, grown to OD₆₀₀ = 0.6 and frozen in 1 ml aliquots. Genomic DNA was extracted and the Tn insertion sites mapped as previously described (21).

To identify genes that confer a fitness advantage in stationary phase, the *B. ovis* Tn-himar library was inoculated in BB in a 5% CO₂ environment in triplicate at an OD₆₀₀ of 0.0025. An aliquot of each initial culture was collected as the reference time point. Cultures were then grown to stationary phase, with samples harvested throughout the growth curve at OD₆₀₀ of 0.05, 0.12, 0.9 and 2.4. Cells from each sample were pelleted by centrifugation and resuspended in water. Barcodes were amplified from approximately 1.5 x 10⁸ cells per PCR reaction (see Table S1) with primers that both amplified the barcodes and added indexed adaptors (21). Amplified barcodes were then pooled, purified and sequenced on an Illumina HiSeq 4000.

Fitness scores for each gene were calculated following the protocol of Wetmore and colleagues (21) using scripts available at https://bitbucket.org/berkeleylab/feba. Genes for which 2 out of 3 samples had at least one timepoint with a t-like statistical significance score (21) ≥ |4| were included in subsequent analyses. A heat map of fitness scores of genes passing this filter is shown in Fig. S1 and the raw fitness data are in Data Set 1.

Finally, we averaged the fitness values of the three replicates and kept mutants that an average fitness score ≥ |1| in at least one time point. Mutants in this group with a standard deviation ≥ 0.75, were manually inspected and extreme outlier points were removed from a total of six genes. The genes with adjusted average and standard deviation values are shown in red in Data Set 1. Heat map of averaged fitness values is shown in Fig. 1.

Tissue culture

All tissue culture cells were grown at 37°C with 5% CO₂ supplementation. THP-1 cells (ATCC TIB-202) were cultured in Roswell Park Memoriam Institute medium (RPMI 1640, Gibco) + 10% Fetal Bovine Serum (FBS, Fisher Scientific). The RAW 264.6 (ATCC TIB-71) and the OA3.ts (ATCC CRL-6546) cells were grown in Dulbecco’s Modified Eagle Medium (DMEM, Gibco) supplemented with 10% FBS.

Infection assays

THP-1 cells were seeded at a concentration of 10⁵ cells/well in 96-well plates and phorbol myristate acetate (PMA) at final concentration of 50 ng/µl was added to induce differentiation into macrophage-like cells for 48-96 h prior to infection. OA3.ts cells were seeded at a density of 5 x 10⁴ cells/well in 96-well plates for 24 h prior to infection. *B. ovis* cells were resuspended from a 48 hr old plate in RPMI + 10% FBS or DMEM + 10% FBS and added to tissue culture plates on the day of infection at multiplicity of infection (MOI) of 100 for THP-1, and at an MOI of 1000 for OA3.ts cells. When infecting with the complementation strains carrying the pSRK plasmid, the *Brucella* strains were struck on TSA blood plates with Kan and IPTG 48 hrs prior to infection, and 2 mM IPTG was added to the tissue culture media throughout the duration of the experiment. Plates were spun for 5 min at 150 x g and incubated for 1 hr at 37 °C in 5% CO₂. Fresh media was supplied containing 50 µg/ml of gentamicin and incubated for another hour. Cells were then washed once with PBS and once in H₂O and then lysed with H₂O for 10 min RT at 2 hrs, 24 hrs, and 48 hrs post infection. Lysates were serially diluted, spotted on TSA blood plates and incubated at 37 °C in 5% CO₂ for 48 hrs to enumerate CFUs. Experiments were repeated at least 3 times with three technical replicates.

Acknowledgements

We thank David Hershey for providing critical constructive feedback and help with the RB Tn-himar library experiment, as well as Crosson lab members and Josh Lensmire for helpful discussion. This work was funded through the NIH Training Grant T32 GM007197 (L.M.V.), NIH grant R35 GM131762 (S.C.), and the Gallo Global Health Fellowship Program (L.M.V.).

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