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SdrA, an NADP(H)-regenerating enzyme, is crucial for *Coxiella burnetii* to resist oxidative stress and replicate intracellularly

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Running title: *sdrA* is required for *C. burnetii* pathogenesis

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SUMMARY

Coxiella burnetii, the causative agent of the zoonotic disease Q fever, is a Gram-negative bacterium that replicates inside macrophages within a highly oxidative vacuole. Screening of a transposon mutant library suggested that *sdrA,* which encodes a putative short chain dehydrogenase, is required for intracellular replication. Short chain dehydrogenases are NADP(H)-dependent oxidoreductases, and SdrA contains a predicted $NADP^+$ binding site, suggesting it may facilitate NADP(H) regeneration by *C. burnetii,* a key process for surviving oxidative stress. Purified recombinant 6xHis-SdrA was able to convert $NADP^+$ to NADP(H) *in vitro*. Mutation to alanine of a conserved glycine residue at position 12 within the predicted NADP binding site abolished significant enzymatic activity. Complementation of the *sdrA* mutant (sdrA::Tn) with plasmid-expressed SdrA restored intracellular replication to wild type levels, but expressing enzymatically inactive G12A_SdrA did not. The *sdrA*::Tn mutant was more susceptible *in vitro* to oxidative stress, and treating infected host cells with L-ascorbate, an anti-oxidant, partially rescued the intracellular growth defect of *sdrA*::Tn. Finally, stable isotope labelling studies demonstrated a shift in flux through metabolic pathways in *sdrA*::Tn consistent with the presence of increased oxidative stress, and host cells infected with *sdrA*::Tn had elevated levels of reactive oxygen species compared to *C. burnetii* NMII.

Key words: *Coxiella burnetii*, oxidative stress, NADP(H) metabolism, short chain dehydrogenase

INTRODUCTION

Coxiella burnetii is a Gram-negative pathogenic bacterium that grows within the mammalian host in an acidic vacuole, known as the *Coxiella*-containing vacuole (CCV), thriving within this proteolytic and oxidative environment (Moffatt *et al.*, 2015). Humans acquire infection through inhalation of contaminated aerosols and show clinical signs ranging from asymptomatic infection to life-threatening systemic infection (van Loenhout *et al.*, 2015). The primary livestock reservoirs for *C. burnetii* are ruminants, in which infection is often subclinical, but bacteria shed by these animals, particularly in birthing fluids, contaminate the environment (Delsing *et al.*, 2012) leading to a significant threat to public health. This was demonstrated by the Q fever outbreak in the Netherlands from 2007 to 2011, the largest reported to date, with more than 4000 human cases and the culling of thousands of animals (van Asseldonk *et al.*, 2013).

In natural infection *C. burnetii* has a strong tropism for alveolar macrophages, but it can also infect a wide range of both phagocytic and non-phagocytic cells (Voth *et al.*, 2007). The bacterium undergoes a biphasic lifecycle, differentiating from the environmentally stable small cell variant (SCV) into a replicating large cell variant (LCV) inside the CCV (Moffatt *et al.*, 2015). *C. burnetii* possesses a Type IV secretion system, the Dot/Icm system, which translocates at least 130 effectors into the host cell (Moffatt *et al.*, 2015) and is required for intracellular replication (Beare *et al.*, 2011, Carey *et al.*, 2011, Newton *et al.*, 2014). Screening a transposon mutant library for replication within HeLa cells suggested that, in addition to the Dot/Icm apparatus and effectors, a number of other bacterial factors are important in pathogenesis (Newton *et al.*, 2014), including genes encoding metabolic enzymes, such as *nadB,* which encodes an enzyme in the *de novo* NAD biosynthetic pathway that we recently confirmed was required for intracellular replication (Bitew *et al.*, 2018).

In the same transposon mutant library screen a *cbu1276* transposon mutant was identified as attenuated for intracellular replication. This gene encodes a putative short chain dehydrogenase, a class of enzymes that are NADP(H)-dependent oxidoreductases, for which we propose the gene name *sdrA* (short chain dehydrogenase reductase A). SdrA contains a predicted $NADP^+$ binding site, suggesting it may facilitate NADP(H) regeneration by *C. burnetii.* NADP(H) is an essential reducing agent in all living organisms and is involved in a variety of biological reactions, and is particularly important in anti-oxidative defence mechanisms because of its reducing power (Singh *et al.*, 2008). *C. burnetii* lacks the oxidative branch of the pentose phosphate pathway, a main source of NADP(H) regeneration for many organisms, and it is unclear how, in the absence of this pathway, sufficient NADP(H) is generated (Omsland *et al.*, 2011b). To examine if SdrA plays a key role in intracellular replication due to its ability to regenerate the NADP(H) crucial for resistance to oxidative stress inside the CCV, recombinant SdrA was expressed and purified and its enzymatic activity characterised. The *sdrA* transposon mutant was genetically complemented with plasmid expressing both active and inactive SdrA and intracellular replication assays were carried out using both epithelial and macrophage-like cells. Furthermore, the ability of *C. burnetii* to resist oxidative stress *in vitro* was investigated, as was the effect of anti-oxidant treatment on replication of the *sdrA* mutant inside macrophage-like cells. Finally, sophisticated metabolomic techniques were utilised to examine the metabolic shifts occurring in the bacterium in the absence of SdrA, and a fluorogenic probe used to detect and quantify the levels of reactive oxygen species inside infected and unifected host cells.

RESULTS

sdrA is required for efficient intracellular replication and CCV formation by *C. burnetii*

To confirm that *sdrA* is required for intracellular replication inside HeLa cells, the *sdrA* mutant (containing a transposon inserted 205 base pairs into the open reading frame) was clonally isolated and genetically complemented with a *C. burnetii* plasmid expressing full length SdrA with an N-terminal 3xFLAG tag (pFLAG:*sdrA)*. Expression of full-length protein of the expected size by the complemented mutant was confirmed using immunoblotting (Figure S1). Intracellular growth assays carried out in HeLa CCL2 cells revealed that the *sdrA* mutant displayed a severe replication defect, with only an 11-fold increase in genome equivalents at seven days post infection, compared to *C. burnetii* NMII that showed a 330-fold increase (Figure 1A). The complemented mutant increased by 265-fold at day seven, confirming that *sdrA* is required for efficient intracellular replication (Figure 1A). Immunofluorescence microscopy revealed that the *sdrA* mutant formed a much smaller vacuole (stained green) containing fewer bacteria (stained red), when compared to either *C. burnetii* NMII or the complemented mutant (Figure 1B).

SdrA reduces $NADP^+$ to NADP(H) *in vitro*

To investigate if SdrA can convert $NADP^+$ to NADP(H), recombinant 6×His-SdrA was expressed in and purified from *Escherichia coli* JM109 and enzyme assays were carried out *in vitro*. Expression of protein of the correct size was confirmed by immunoblotting with anti-His antibody (Figure S2A). Ethyl (S)-(−)-4-chloro-3-hydroxybutyrate, a common substrate of short chain dehydrogenases (Xue *et al.*, 2011), was utilised in the assay with $NADP^+$ as the cofactor. Following addition of recombinant enzyme an increase in absorbance at 340nm over time was observed (Figure 2), indicating that SdrA was

converting $NADP^+$ to NADP(H). In contrast, no change in absorbance was observed when mock purification was substituted for 6×His-SdrA in the assay mixture.

NADP(H) regeneration by SdrA is essential for efficient intracellular replication of *C. burnetii*

To examine if the enzymatic function of SdrA is required for *C. burnetii* intracellular replication, a conserved glycine residue at position 12 that we identified as likely to be necessary for $NADP^+$ binding (Hua *et al.*, 2014), was mutated to alanine. Recombinant 6×His-G12A was then expressed in and purified from *E. coli* JM109, with expression of protein of the correct size confirmed by immunoblotting with anti-His antibody (Figure S2B). When this mutant derivative protein 6×His-G12A was used in the *in vitro* enzyme assay minimal change in absorbance at 340nm was observed (Figure 2), confirming that mutation of the predicted $NADP^+$ binding site abolished significant enzymatic activity. To determine if the NADP(H) generating activity of SdrA is necessary for *C. burnetii* to replicate intracellularly, the *sdrA* mutant was complemented with plasmid expressing full-length 3xFLAG-tagged G12A-SdrA (Figure S1) and qualitative and quantitative intracellular replication assays using the macrophage-like THP-1 cells were performed. As in HeLa cells, the *sdrA* mutant was significantly attenuated in growth, with only a 401-fold increase in genome equivalents at day seven (Figure 3A), compared to *C. burnetii* NMII that increased by 6.2 $\times 10^5$ fold. Complementation of the *sdrA* mutant with the G12A-SdrA enzyme that lacks significant NADP(H) regenerating activity resulted in a similar phenotype to the *sdrA* mutant, with only a 913-fold increases in genome equivalents at day seven. In contrast, as in HeLa cells, complementation with the plasmid expressing native SdrA restored replication to *C. burnetii* NMII levels. Immunofluorescence microscopy also demonstrated that the G12A_SdrA complemented mutant, compared to *C. burnetii* NMII, produced smaller CCVs with far fewer bacteria, similar to the original *sdrA* mutant (Figure 3B). These findings confirm that

enzymatically active SdrA, and therefore NADP(H) regeneration by this enzyme, is required for intracellular replication of *C. burnetii*.

Disruption of *sdrA* results in greater susceptibility to oxidative stress

To investigate if the loss of NADP(H) regeneration in the absence of SdrA resulted in increased susceptibility to oxidative stress, *in vitro* hydrogen peroxide sensitivity assays were performed. *C. burnetii* strains were cultured in medium containing varying concentrations of H_2O_2 for seven days before the genome equivalents were quantified by qPCR and compared to untreated controls, as an indirect measure of survival of the H_2O_2 exposure at day zero (as this compound is inherently unstable and breaks down in culture medium rapidly). At a low concentration (50 µM) of H_2O_2 no strains were susceptible to oxidative stress, with no significant difference in replication between untreated and treated cultures. At 500µM H_2O_2 *C. burnetii* NMII and *sdrA*::Tn pFLAG:*sdrA* still displayed minimal susceptibility to oxidative stress compared to the untreated controls (Figure 4A), with no significant difference in genome equivalents between treated and untreated strains at day seven. In contrast, treatment of *sdrA*::Tn with 500µM H_2O_2 resulted in a significant reduction in replication at day seven when compared to the untreated control (Figure 4A). However, *C. burnetii* NMII and *sdrA*::Tn pFLAG:*sdrA* were still susceptible to oxidative stress induced by hydrogen peroxide, as treatment with a higher concentration (1mM H_2O_2) resulted in a statistically significant decrease in genome equivalents of all strains at day seven after treatment (Figure 4B) compared to the untreated controls, with the greatest decrease in replication observed for *sdrA*::Tn at this higher concentration. Taken together, these results show that SdrA is required for resistance to oxidative stress *in vitro*. This data also demonstrated that *sdrA*::Tn displays no replication defect in axenic media, as similar replication was observed for the untreated cultures of all strains (Figure S3).

Treatment with the anti-oxidant L-ascorbic acid can partially rescue the intracellular replication defect of the *sdrA* mutant

As the *sdrA* mutant is more susceptible to oxidative stress *in vitro*, we investigated whether the commonly available anti-oxidant L-ascorbate affected growth of this strain inside host cells. An intracellular replication assay was performed in THP-1 cells using *C. burnetii* NMII and the *sdrA* mutant. When the *sdrA* mutant was used to infect THP-1 cells grown in medium supplemented with L-ascorbate there was a significant increase in replication at day seven, with a 4×10^4 fold increase in the L-ascorbate-positive condition compared to only a 280-fold increase for the *sdrA* mutant in the L-ascorbate-negative condition (Figure 5A). Immunofluorescence images also showed that the *sdrA* mutant grown inside THP-1 cells in L-ascorbate-positive conditions formed a larger CCV with numerous bacteria, similar to the *C. burnetii* NMII (Figure 5B). Overall, treatment with anti-oxidant significantly improved the ability of the *sdrA* mutant to replicate inside host cells, further supporting the key role of SdrA in facilitating resistance to oxidative stress.

The *sdrA* mutant displays changes in metabolic flux consistent with the presence of increased oxidative stress *in vitro*

NADP(H) is an important coenzyme in a number of metabolic reactions (Spaans *et al.*, 2015). In order to investigate the effects of limited NADP(H) regeneration on *C. burnetii* metabolism more generally, gas chromatography-mass spectrometry (GC/MS) was used to compare the polar metabolite profiles of *C. burnetii* NMII*,* the *sdrA* mutant and the complemented *sdrA* mutant grown axenically for 7 days. A total of 80 metabolites were identified using standard retention time and mass spectra by comparing with in house authentic standards. The data was median normalised to account for any differences in

cell number, and statistically analysed to compare the level of abundance of metabolites between *C. burnetii* NMII and the *sdrA* mutant, and the *sdrA* mutant and the complemented mutant. Both the hierarchical clustering (Figure S4) and principal component analyses (Figure S5) showed clustering of the *sdrA* mutant replicates completely separate to the *C. burnetii* NMII and complemented mutant replicates. In contrast, the observed overlap between *C. burnetii* NMII and complemented mutant replicates indicated that the abundance of metabolites in these two strains was relatively similar, and pairwse comparision showed no significant differences in metabolite abundances between *C. burnetii* NMII and the complemented *sdrA* mutant.

Comparison of *C. burnetii* NMII and the *sdrA* mutant identified 31 metabolites that were significantly different in abundance ($P < 0.05$, Benjamini-Hochberg [BH]-adjusted t-test) (Figure 6), varying by 3.9 to -4.02 $\log_2$ fold change, whereas pairwise comparison of complemented mutant versus *sdrA* mutant revealed 29 metabolites to be significantly different in abundance ($P < 0.05$, Benjamini-Hochberg [BH]-adjusted t-test) (Figure 6), varying by a similar $\log_2$ fold change range of 3.9 to -4.1, with 25 metabolites common to both groups. Metabolites related to central carbon metabolic pathways such as the tricarboxylic acid cycle (TCA), glycolysis, the pentose phosphate pathway (PPP) and amino acid metabolism showed statistically significant differences that were, in general, consistently different in the direction of fold change, being either elevated in only the mutant or in both the *C. burnetii* NMII and complemented mutant (Figure 6). For example, the TCA cycle intermediate malate was increased in both *C. burnetii* NMII and the complemented mutant, whereas other TCA cycle metabolites such as citric acid and aconitic acid were elevated in the *sdrA* mutant (Figure 6). The pentose phosphate pathway intermediate, ribose 5-phosphate, was decreased in the mutant compared to both *C. burnetii* NMII and the complemented mutant. Overall, the steady state profiling results suggested that loss of SdrA had a significant impact of central carbon metabolism. To investigate this finding further, stable

isotope labelling using [^{13}C] glucose was performed to analyse changes in metabolic fluxes in the absence of SdrA. Strains were cultured to late logarithmic phase (day 6) in axenic medium before labelling. All strains were able to efficiently internalise [^{13}C] glucose after one hour of labelling and label was incorporated into intermediates of glycolysis, the TCA cycle and certain amino acids (Figure 7). Significantly lower label incorporation was detected in intermediates of glycolysis in the *sdrA* mutant compared to both *C. burnetii* NMII and the complemented strain. For example, 78.5% label enrichment was observed for glucose-6-phosphate (G6P) in *C. burnetii* NMII and 77.3% in the complemented mutant, compared to only 46.7% enrichment in G6P in the *sdrA* mutant. Similar significant decreases in label incorporation were observed in the *sdrA* mutant in fructose-6-phosphate and in metabolites derived from intermediates of glycolysis such as glycerol-3-phosphate, demonstrating lower flux through this pathway in the absence of SdrA.

Pyruvate, the end product of glycolysis, can feed into a number of metabolic pathways, including the TCA cycle and alanine synthesis. Label incorporation into alanine was significantly reduced in the *sdrA* mutant (0.7%) compared to both *C. burnetii* NMII (18.4 %) and the complemented mutant (16.5 %), demonstrating decreased flux through this pathway. Striking differences were also observed in the TCA cycle. Label was incorporated into malate in *C. burnetii* NMII (20.2%) and the complemented mutant (19.7%) at much higher levels compared to the *sdrA* mutant (3.8%). Label enrichment in succinate for *C. burnetii* NMII (20.2%) and the complemented mutant (19.2%) was also significantly higher compared to the *sdrA* mutant (5.6%), as was label enrichment in fumarate, with values of 15.3%, 17.7% and 5.2% for *C. burnetii* NMII, the complemented mutant and the *sdrA* mutant respectively (Figure 7). Analysis of the mass isotopologue distribution reveals a predominantly +2 labelling pattern in TCA intermediates for all strains, which indicates that carbon is predominantly entering the TCA cycle via acetyl-CoA (Figure S6). Overall this data demonstrates significant changes

in flux through central carbon metabolism of *C. burnetii* in the absence of SdrA, with a decrease in flux through metabolic pathways associated with production of the pro-oxidant NAD(H), such as glycolysis and the TCA cycle.

Levels of reactive oxygen species (ROS) are higher in THP-1 cells infected with *C. burnetii* compared to uninfected cells, and the loss of SdrA significantly increases ROS levels

There is evidence that *C. burnetii* reduces generation of ROS by infected host cells, and therefore there is some uncertainty as to how oxidative an environment the CCV actually is (Mertens *et al.*, 2012). To investigate this further, and thus clarify if the role of SdrA is related to oxidative defence, the levels of ROS in infected THP-1 cells were compared to those present in uninfected THP-1 cells. Quantification of ROS levels demonstrated that not only are ROS levels elevated in infected THP-1 cells compared to uninfected controls, suggesting that the CCV is an oxidative environment, but significantly higher levels of ROS were detected in THP-1 cells containing the *sdrA* mutant compared to host cells containing either *C. burnetii* NMII or the complemented *sdrA* mutant (Figure 8), further strengthening the hypothesis that SdrA is crucial for defence against oxidative stress.

DISCUSSION

Exposure to oxidative stress can damage DNA, membranes and proteins, and most organisms have therefore evolved a number of mechanisms to protect against the deleterious effects of oxidative stress (Ezraty *et al.*, 2017). The defence mechanisms utilised by *C. burnetii* are only partly characterised and are predominantly inferred from genome annotation (Mertens *et al.*, 2012). Two key classes of enzymes involved in oxidative defence are the oxidoreductases thioredoxins (Trxs) and glutaredoxins (Grxs). Trxs reduce oxidised cysteine residues in damaged proteins to the thiol state and are themselves oxidised. Regeneration of reduced Trxs requires thioredoxin reductase, which utilises NADP(H) as a cofactor (Ezraty *et al.*, 2017). The *C. burnetii* genome contains an annotated thioredoxin reductase (CBU2087), and reduced availability of NADP(H) will therefore impair this oxidative defence mechanism. A Trx peroxidase (CBU1706) is also annotated on the *C. burnetii* genome (Seshadri *et al.*, 2003). This enzyme catalyzes the conversion of H_2O_2 to H_2O, with concurrent oxidation of Trx (Berggren *et al.*, 2001, Ihnatko *et al.*, 2012). Furthermore, *C. burnetii* expresses a DNA-binding peroxiredoxin (CBU0963) that displays peroxidase activity that is also dependent on thioredoxin/thioredoxin reductase (Hicks *et al.*, 2010), and therefore also requires an adequate supply of NADP(H). Inadequate availability of reduced Trxs for these enzymatic reactions, due to insufficient NADP(H), may explain the increased sensitivity of the *C. burnetii sdrA* mutant to oxidative stress induced by H_2O_2 as well as its greatly reduced intracellular replication. Methionine sulfoxide reductase (MsrA, CBU1306) is another enzyme involved in oxidative defence that requires reduced Trx (Mertens *et al.*, 2012, Ezraty *et al.*, 2017), and inadequate NADP(H) will therefore impair this mechanism as well. *E. coli* mutants that lack Trxs can still survive oxidative stress due to the presence of Grxs (Ezraty *et al.*, 2017). Grxs also facilitate the reduction of oxidised cysteines in proteins damaged by oxidative stress. The oxidised Grx is then reduced by glutathione (a low molecular weight thiol that is a key anti-oxidant

in many organisms). Oxidised glutathione is then reduced by glutathione reductase, which is an NADP(H)-dependent enzyme (Ezraty *et al.*, 2017). Inadequate levels of NADP(H) will therefore also prevent this repair system functioning properly. The genome of *C. burnetii* encodes glutaredoxin (*grxC;* CBU1520) and the genes required for glutathione synthesis (Mertens *et al.*, 2012), although glutathione reductase itself is not currently annotated on the *C. burnetii* genome. *C. burnetii* also possesses several non-NADP(H) dependent mechanisms of oxidative stress including two superoxide dismutases and catalase (KatE), all capable of directly detoxifying ROS, which may explain why the *sdrA* mutant can still replicate to low levels. It is worth noting, however, that strains lacking functional catalase are still able to cause disease (Mertens *et al.*, 2012). Overall, although *C. burnetii* appears to have a number of mechanisms to avoid and cope with oxidative stress (Mertens *et al.*, 2012), the inability of the *sdrA* mutant to efficiently replicate inside host cells suggests that NADP(H)-related oxidative defence mechanisms are critical. The partial rescue of the intracellular replication defect of the *sdrA* mutant by the addition of L-ascorbate suggests this anti-oxidant is trafficked to the CCV by the host cell, and that exogenous anti-oxidants can partly overcome the loss of NADP(H), perhaps through their activity in scavenging free radicals and reducing oxidative damage (Liang *et al.*, 2001, Saffi *et al.*, 2006). However, given the complex nature of defence mechanisms against oxidative stress it is not unexpected that complete rescue of the intracellular growth defect was not observed.

As NADP(H) is crucial for *C. burnetii* resistance to oxidative stress inside host cells, it is perhaps surprising that *C. burnetii* lacks the oxidative PPP, a major source of NADP(H) in many organisms (Omsland *et al.*, 2011b, Spaans *et al.*, 2015). Other enzymes of central carbon metabolism that may utilise NADP(H) as a co-factor include isocitrate dehydrogenase (ICD), malic enzyme and glutamate dehydrogenase. Label incorporation into isocitrate/citrate could not be detected due to the influx of extracellular citrate from the media, which diluted any endogenous citrate/isocitrate signal, and 2-

oxoglutarate is not detectable using GC/MS, so the activity of ICD is not readily apparent. Conversion of isocitrate to 2-oxoglutarate may produce NADP(H), as previous work suggests that *C. burnetii* ICD utilises NADP(H) as a cofactor rather than NAD(H) (Van Nguyen *et al.*, 1999). However, the significantly lower label incorporation into all detected TCA cycle intermediates in the *sdrA* mutant demonstrates an overall decreased flux through this pathway, suggesting that *C. burnetii* is unable to significantly upregulate isocitrate dehydrogenase to compensate for the reduced NADP(H) regeneration in the absence of SdrA. The observed decreased flux through the TCA cycle is also consistent with an oxidative stress response by the *sdrA* mutant, as overall the energy produced by acetyl-CoA catabolism via the TCA cycle results in production of the potent pro-oxidant NAD(H) and promotes a highly oxidative cellular environment. Downregulation of the TCA cycle has been reported by other bacteria undergoing exogenous oxidative stress (Singh *et al.*, 2007). Furthermore, incorporation of label into alanine from pyruvate by alanine dehydrogenase, a reaction that produces NAD(H), was almost zero for the *sdrA* mutant, whereas alanine in *C. burnetii* NMII and the complemented *sdrA* mutant were 15-20% labelled, demonstrating additional metabolic shifts by the *sdrA* mutant to reduce endogenous oxidative stress. These metabolic changes may also reduce the fitness of the *sdrA* mutant, although no difference in axenic growth was observed. However, we demonstrated recently (Kuba *et al*., 2019) that the metabolic pathways favoured by intracellular bacteria differ from those utilised axenically, and so the observed metabolic changes may also contribute to the decreased replication of the *sdrA* mutant inside host cells. Nonetheless, the significantly increased ROS observed inside THP-1 cells infected with the *sdrA* mutant suggests that SdrA has a critical role in defense against oxidative stress. The [^{13}C]-glucose labeling experiments were broadly consistent with a recent study examining utilisation of [^{13}C]-glucose by *C. burnetii* (Häuslein et al., 2017), with incorporation into glycolytic intermediates such as G6P (despite the absence of an annotated glucokinase (Hackstadt et al., 1981)).

The significantly lower incorporation of [^{13}C]-label from glucose into glutamate in the *sdrA* mutant indicates reduced activity of glutamate dehydrogenase in converting 2-oxoglutarate to glutamate, a reaction that consumes NADP(H), demonstrating another shift in metabolic flux related to insufficient NADP(H) in the absence of SdrA.

The substrate for SdrA *in vivo,* and why *C. burnetii* evolved to utilise this enzyme rather than the oxidative PPP to regenerate NADP(H), is still unclear. Short chain dehydrogenase enzymes have broad substrate specificity (Brock *et al.*, 2008, Kallberg *et al.*, 2010) and common substrates include alcohols such as ethyl (S)-(−)-4-chloro-3-hydroxybutyrate (Xue *et al.*, 2011). Analysis of the current metabolic map of *C. burnetii* within the KEGGS database (https://www.genome.jp/kegg/) does not reveal any obvious enzyme gaps in currently annotated pathways that SdrA may fill. Sequence analysis indicates that *C. burnetii* SdrA belongs to the class IV short chain dehydrogenases (Hua *et al.*, 2014), but this classification relates to the NADP binding site and does not assist in identifying likely substrates.

The identification of SdrA and the characterisation of its function answers a key question regarding *C. burnetii* metabolism, as it identifies a novel enzyme that may compensate significantly for the absence of the oxidative PPP. Although other enzymes such as isocitrate dehydrogenase, malic enzyme and glutamate dehydrogenase can also regenerate NADP(H), this study suggests that these enzymes are not a critical source of reducing equivalents for *C. burnetii.* Furthermore, this study demonstrates that SdrA is a critical enzyme for *C. burnetii* to regenerate sufficient NADP(H) to resist the oxidative environment of the CCV and replicate inside host cells, a crucial process for the bacterium to cause disease.

EXPERIMENTAL PROCEDURES

Bacterial strains, cell lines and growth conditions

Plaque-isolated *C. burnetii* Nine Mile phase II (NMII) RSA 439 was axenically cultured in ACCM-2 broth or on ACCM-2 agarose plates in a humidified atmosphere of 5% CO_2 and 2.5% O_2 at 37^{o}C. The selection of transposon mutants and plasmids were carried out using chloramphenicol (3µg/ml) and kanamycin (350 µg/ml) as required. *Escherichia coli* XL1-Blue super competent cells (Agilent Technologies) were used for site directed mutagenesis. *E. coli* DH5a strain was used for cloning and *E. coli* JM109 for recombinant protein expression and purification. *E. coli* strains were cultured in Luria-Bertani medium with chloramphenicol (25 µg/ml), kanamycin (100 µg/ml) and ampicillin (100 µg/ml) added as required for plasmid selection. Super Optimal broth with Catabolite repression (SOC) was used for mutagenesis and transformation of XL1-Blue super competent cells. Macrophage like THP-1 cells and HeLa CCL2 cells were grown and maintained in Roswell Park Memorial Institute (RPMI) medium with 5 or 10% fetal calf serum (FCS) and Dulbecco's Modified Eagle's Medium (DMEM) with 5 or 10% fetal calf serum (FCS) at 37°C in 5% CO_2 respectively.

Clonal isolation of the *sdrA* mutant

The previously generated *C. burnetii sdrA* transposon mutant (Newton *et al.*, 2014) was clonally isolated using two layered ACCM-2 agarose plates containing chloramphenicol (3µg/ml) as described previously (Omsland *et al.*, 2011a). Isolated mutant clones were screened by PCR using *sdrA* specific and transposon primer combinations (Table 1) to confirm the presence of the transposon disrupting *sdrA*.

Genetic complementation of the *sdrA* mutant

To genetically complement the *sdrA* mutant, full length *sdrA* was amplified using specific primers containing engineered Sal1 restriction sites (Table 1) and cloned into the *C. burnetii* complementation vector pJB-Kan:3xFLAG (Beare, 2012). Insertion of *sdrA* in the correct orientation was confirmed by DNA sequencing. The pFLAG:*sdrA* construct was introduced into the *sdrA* transposon mutant as described previously (Newton *et al.*, 2014). Briefly, *C. burnetii* NMII grown in ACCM-2 was pelleted by centrifugation at 15000×g at 4 °C for 15 minutes and the bacterial pellet washed in 20 ml of ice-cold 10% glycerol. The bacteria were pelleted again and resuspended in 100 µl of ice-cold 10% glycerol. Ten µg of plasmid DNA was electroporated into the cells at 18 kV, 500 Ω, and 25 µF before resuspension in RPMI and transfer to ACCM-2 for overnight recovery followed by addition of kanamycin. Transformants were plated on solid ACCM-2 agarose plates and incubated for 6 days at 5% CO_2 and 2.5% O_2 at 37^{o}C. Selected colonies were expanded into 24 well plates in ACCM-2 and screened by immunoblotting for expression of FLAG-SdrA (Figure S1). For immunoblotting, bacterial whole cell lysate was analysed on 12.5% SDS-PAGE polyacrylamide gel as previously described (Schägger *et al.*, 1991) and transferred to nitrocelulose membrane that was then probed with mouse anti-Flag IgG antibody (Sigma) diluted at 1/1000 in 1 % (w/v) skim milk in PBS-T and anti-mouse IgG HRP (GE Healthcare) diluted to 1/3000 in 1% (w/v) skim milk in PBS-T before signal development using chemiluminescence.

Intracellular replication assays in HeLa cells and THP-1 cells

HeLa CCL_2 cells were seeded into 24 well plates at a density of 5 x 10^4 with or without glass coverslips one day before infection. *C. burnetii* strains grown in axenic medium for seven days were harvested and resuspended in DMEM + 5% FCS and the bacteria quantified by qPCR amplifying *ompA,* as described previously (Jaton *et al.*, 2013), to determine an MOI of 50. Cells were infected by *C. burnetii*

diluted in DMEM + 5% FCS and incubated for 4 hours. The media was removed and cells were washed with PBS to remove extracellular bacteria before fresh DMEM + 5% FCS was added to all well plates except Day 0. The cells were lysed in H_2O and lysate collected at Day 0, 1, 3, 5 and 7 and *C. burnetii* genome equivalents quantified by qPCR using *ompA* specific primers. Immunofluorescence microscopy was carried out as described previously (Bitew *et al.*, 2018). Three days post-infection cells from replicate plates with coverslip were fixed with 4% paraformaldehyde. The cells were blocked and permeabilised with 2% (w/v) BSA and 0.05% (v/v) saponin in PBS for 1 hour. Primary antibodies rabbit anti-*Coxiella* and mouse anti-LAMP1 were diluted in blocking buffer at 1/10000 and 1/500 respectively and used to stain the cells, with secondary antibodies comprising anti-rabbit AlexaFluor 568 and anti-mouse AlexaFluor 488 antibodies diluted in blocking buffer at 1/3000. Before mounting on slides DAPI diluted at 1/10000 in PBS was used to stain DNA. The coverslips were mounted using Dako Fluorescent Mounting Medium and images were acquired with Zeiss LSM700, Zeiss LSM710 and Nikon A1R confocal microscopes and analysed using ImageJ.

Quantitative and qualitative replication assays were carried out in THP-1 cells in a similar manner as for the HeLa cells, except that THP-1 cells were seeded at a density of 5×10^5 cells per well in 24 well plates, and to induce differentiation into macrophage-like cells the THP-1 cells were treated with 10 nM phorbol 12-myristate 13-acetate (PMA) 3 days prior to infection. In order to determine the effect of L-ascorbate on intracellular replication, 0.025 mM L-ascorbate was added to the THP-1 cell medium at day 0.

Site directed mutagenesis of SdrA

Bioinformatics analysis revealed that proteins containing predicted $NADP^+$ binding sites have a common and functionally conserved glycine residue (Hua *et al.*, 2014). In *C. burnetii* this Gly at position

12 was mutated to Ala by site directed mutagenesis using the QuickChange Site-Directed Mutagenesis Kit (Agilent Technologies) on *C. burnetii sdrA* cloned into pGEM-T vector, as per the manufacturer's instructions (Promega). The primers used to introduce the mutation are listed in Table 1. DNA sequencing was used to confirm the correct mutation in pGEM-G12A-*sdrA*.

Expression and purification of recombinant SdrA and G12A_SdrA

Primers specific to *sdrA* (Table 1) were designed for amplification and cloning of both *sdrA* and G12A_*sdrA* into pQE-30 with an N-terminal 6×His-tag using engineered BamH1 and PstI restriction sites. Genomic *C. burnetii* DNA was used as template to amplify *sdrA* whereas G12A_*sdrA* was amplified from pGEM-G12A-*sdrA.* Constructs were transformed into *E. coli* JM109 and protein expression induced for 3 hours by 1 mM isopropyl-beta-D-thiogalactopyranoside when the optical density of the culture reached 0.8. The cells were harvested by centrifugation at 7700 × g for 10 min at 4°C and resuspended in 25 ml ice-cold PBS. Lysozyme at a final concentration of 1 mg/ml and 1 mM phenylmethylsulfonyl fluoride (PMSF) were added and incubated overnight before lysing the cells by sonication. Triton X- 100 at a final concentration of 1 % was added and incubated on ice for 30 minutes. Protein fractions were separated by centrifugation and purified by affinity chromatography using 50% Nickel-Nitrilotriacetic acid (Ni-NTA) slurry (QIAGEN) following the manufacturer's instructions. Dialysis tubing cellulose membrane (Sigma) was used to dialyse the purified protein against buffer containing 50 mM NaH_2PO_4, 300 mM NaCl and 10 mM imidazole and protein concentration measured by Pierce™ BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer's instruction.

***In vitro* short chain dehydrogenase assays**

To determine short chain dehydrogenase enzyme activity, *in vitro* assays were carried out using 1.5% ethyl (S)-(−)-4-chloro-3-hydroxybutyrate (v/v), 0.5 mM $NADP^+$ and 4.5 µg of recombinant protein (6×His-SdrA, 6×His-G12A-SdrA, or equivalent volume of mock purification) in 50 mmol/L glycine - sodium hydroxide buffer (pH 10.5). The change in absorbance was measured at 340 nm at 1 minute interval for 13 minutes using a spectrophotometer. Each enzyme assay was repeated three times.

Induction of oxidative stress

C. burnetii strains were inoculated in ACCM-2 at 1 x 10^4 genome equivalents ml^{-1} and treated with either 500 µM or 1 mM hydrogen peroxide (H_2O_2) before incubation for 6 days at 5% CO_2 and 2.5% O_2 at 37 °C. Untreated control cultures were also incubated under the same conditions for each strain. *C. burnetii* genome equivalents were determined by qPCR for *ompA* gene 6 days post-treatment. Each experiment was repeated three times.

Metabolic quenching and extraction of polar metabolites

Six biological replicates of 20 ml axenic cultures of *C. burnetii* NMII strains were grown to late log phase for 6 days and rapidly quenched in an ethanol-dry ice bath at 0°C to stop metabolism. Cells were harvested by centrifugation at 0°C, 1000 × g for 20 minutes following quenching of the cultures. The cells were briefly washed in ice cold PBS and centrifuged again at 0°C, 1000 × g for 20 minutes. The pellet was resuspended in 1ml of ice cold PBS and transferred to prechilled 1.5 ml microcentrifuge tubes for centrifugation at 0°C at 23000 × g for 15 minutes and the pellet stored on ice until extraction. A 400 µL 400 µL aliquot of extraction solution containing 3:1 (v/v) CH_3OH:H_2O with 1 nmol $^{13}C_6$-sorbitol and 10 nmol $^{13}C_5$-,^{15}N-labeled valine as internal standards was added. The cells were lysed by vortexing and freeze-thawing in liquid nitrogen and an ethanol/dry ice bath. Chloroform was added into the

mixture and adjusted $CHCl_3$:CH_3OH:H_2O at the ratio of 1:3:1 (v/v/v) followed by vortexing and incubation for 10 minutes. The supernatant was transferred into a fresh microcentrifuge tube after centrifugation of the mixture at 0°C at 23000 × g for 15 minutes. To induce phase separation the supernatant was adjusted to $CHCl_3$:CH_3OH:H_2O ratio of 1:3:3 (v/v/v) by addition of dH_2O. Following centrifugation the clear upper aqueous phase was collected into a fresh microcentrifuge tube and dried in rotational vacuum concentrator (RVC-2-33; John Morris Scientific) with 30 µL of methanol added to the final drying step. The pulled point inserts were sealed in GC/LC vials and derivatized using methoxyamine (Sigma) and N,O-bistrimethylsilyltrifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS; Thermo Scientific) before analysis of polar metabolites using the Shimadzu GC-QQQ as described previously (Masukagami *et al.*, 2017, Best *et al.*, 2018).

Statistical analysis and comparison of metabolite profiles

Shimadzu Lab Solutions GC-MS browser software was used to identify and quantify metabolites from all chromatograms and the missing data value was imputed by manual peak integration on the spectra from sorted metabolites with the in-house library and collated into a targeted data matrix. Molecular masses of authentic standards from an in-house Metabolomics Australia library and retention times were used for metabolite identification. Log transformed and median normalized data was statistically analysed by the online tool MetaboAnalyst (http://www.metaboanalyst.ca/) and R-script using an R-based statistical analysis package from Metabolomics Australia, as described previously (Masukagami *et al.*, 2017). The level of abundance of metabolites between *C. burnetii* NMII versus the *sdrA* mutant and complemented mutant versus the *sdrA* mutant were compared using unpaired Student's t-test with the Benjamini-Hochberg adjustment to correct for false discoveries, with $P<0.05$ considered significant.

[^{13}C] stable isotope labeling studies

[^{13}C] glucose labeling was carried out by culturing *C. burnetii* as described for the steady state analysis, followed by labeling for 1 hour in ACCM-2 supplemented with 11.11 mM [^{13}C]-glucose. The labeled cultures were quenched and polar metabolites extracted with 1 nmol *scyllo*-inositol as an internal standard and analyzed by GC/MS as described previously (Masukagami *et al.*, 2017). Data analysis was conducted based on the retention time and by comparing the labeled metabolites with unlabeled sample data using DExSI software (Dagley *et al.*, 2018). Percentage labelling of metabolites was calculated following adjustment for naturally occurring stable isotopic background. The data were exported into excel and GraphPad Prism software used for graphing and statistical analysis.

Detection and quantification of ROS levels in THP-1 cells

Three days before infection THP-1 cells were seeded at a density of 5×10^5 cells/well into 24 well plates with 10 mm glass coverslips and treated with 10 nM PMA to induce differentiation into macrophage-like cells. The cells were infected at multiplicity of infection of 25 in RPMI+5% FCS by *C. burnetii* NMII producing mCherry (Beare *et al.*, 2009) and the *sdrA*::Tn and *sdrA*::TnpFLAG:*sdrA* transposon mutants expressing mCherry (Newton *et al.*, 2014). Three days post infection CellROX® Deep Red Reagent (Invitrogen) was added to the cells at a final concentration of 5 µM and incubated for 30 minutes at 37°C. The medium was removed and the cells were washed three times with PBS. Cells were fixed with 4 % paraformaldehyde (PFA) for 15 minutes. Cells were washed with PBS before DAPI diluted at 1/10000 in PBS was used to stain host DNA. Following staining the coverslips were mounted on glass slides using Dako Fluorescent Mounting Medium. The cells were imaged within two hours on a Nikon

A1R confocal microscope and analysed by ImageJ for both qualitative and quantitative data. The images were taken using the same laser settings for all microscopic fields.

The images were taken from three independent experiments and 50 images were captured per stain for each experiment. Quantification of the fluorescence intensity for each CellROX™ stain was carried out using ImageJ. The CellROX™ derived fluorescence was defined as the quantified peak fluorescence at 644 nm minus the background fluorescence. Fluorescence intensity was also quantified from non-infected THP-1 cells. The software displayed fluorescence values in arbitrary units (AU) and they were converted to comma-separated values (CSV) for statistical analysis and graphing in GraphPad Prism. The data was presented as the mean values of 50 images of cells quantified for each stain (Figure 8B). Image selection and quantification was carried out by an investigator blinded to the strains used for infection.

FIGURE LEGENDS

Figure 1. SdrA is required for intracellular replication of *C. burnetii* **A**. HeLa cells were infected by *C. burnetii* strains at a MOI of 50. At days 1, 3, 5, and 7 the fold change in genome equivalents relative to day 0 was determined by qPCR for *ompA*. The *sdrA*::Tn mutant (squares) showed a significant decrease in fold change in genome equivalents ($P<0.05$, paired t-test) at days 5 and 7 as compared to *C. burnetii* NMII (circles) and the complemented mutant (*sdrA*::Tn pFLAG:*sdrA*, triangles). Error bars represent mean $\pm$ standard deviation (n=3). **B.** Confocal images of HeLa cells infected for 3 days with *C. burnetii* NMII, the *sdrA*::Tn mutant and the complemented mutant (*sdrA*::Tn pFLAG:*sdrA*). The cells were fixed with 4% paraformaldehyde and stained with rabbit anti-*Coxiella* (red) and mouse anti-LAMP1 (green) antibodies and DAPI (blue) to stain nucleic acids. The *sdrA*::Tn mutant formed smaller CCVs containing fewer bacteria whereas both *C. burnetii* NMII and the complemented mutant produced a much bigger CCV containing a large number of bacteria. The white asterisks indicate representative CCVs. The images were taken using a Nikon A1R confocal microscope and are representative of three independent experiments. The scale bar represents 10 µm.

Figure 2. SdrA facilitates reduction of NADP to NADP(H) and substituting glycine-12 with alanine results in minimal NADP(H) synthesis. A change in absorbance at 340nm occurs if $NADP^+$ is reduced to NADP(H), with concurrent oxidation of ethyl (S)-(−)-4-chloro-3-hydroxybutyrate. A significant increase in absorbance occurs when 4.5 µg 6xHis-SdrA is present, when compared to the assay carried out using equivalent volume of a mock purification(*P<0.05,* paired t-test), demonstrating that SdrA possesses short chain dehydrogenase activity. Mutation of the NADP binding site of SdrA resulted in minimal change in absorbance, with significantly reduced absorbance for 6xHis-G12A compared to

6xHis-SdrA (*P<0.05,* paired t-test) from the 4 minute timepoint onwards, indicating minimal residual enzymatic activity. Squares indicate recombinant 6xHis-SdrA, open circles represent 6xHis-G12A whereas closed circles are mock purified protein which serve as the negative control. Error bars represent mean ± SD (n=3).

Figure 3. The intracellular replication defect of the *sdrA*::Tn mutant was not restored by complementation with a plasmid encoding the mutant G12A-SdrA protein lacking significant enzymatic activity. **A**. Macrophage-like THP-1 cells were infected with *C. burnetii* strains for seven days and the genome equivalents quantified by qPCR using *ompA* specific primers. The fold change relative to day 0 was determined at days 1, 3, 5, and 7 and presented as mean ±SD of three independent experiments. Both *sdrA*::Tn mutant (closed squares) and *sdrA* mutant complemented with mutant G12A (*sdrA*::Tn pFLAG:G12A ,triangles) showed a small increase in genome equivalents and significant reduction in replication (*P <0.05* paired *t*-test) at day 3, 5 and 7 compared to *C. burnetii* NMII (circles), demonstrating that highly active SdrA is required for normal intracellular replication. In contrast, as for HeLa cells, complementing *sdrA* mutant with active SdrA (*sdrA*::Tn pFLAG:*sdrA*, open squares) restored replication to be equivalent to *C. burnetii* NMII. **B**. Confocal images of THP-1 cells infected with *C. burnetii* strains for 3 days at MOI of 25. The cells were fixed with 4% paraformaldehyde and stained with anti-*Coxiella* (red), anti-LAMP1 (green), and DAPI (blue). Both the *sdrA*::Tn and *sdrA*::Tn pFLAG:G12A produced smaller CCVs containing fewer bacteria whereas both *C. burnetii* NMII and *sdrA*::Tn pFLAG:*sdrA* displayed bigger CCVs containing a large number of bacteria. The images are representative of three experiments. The scale bars represent 10 µm. White asterisks indicate representative CCVs.

Figure 4. SdrA is required for resistance to oxidative stress induced by hydrogen peroxide treatment. *C. burnetii* replication is expressed as a fold change in genome equivalents after *C. burnetii* genomes were quantified by qPCR seven days post treatment with H_2O_2. All three strains displayed no growth defect after treatment with 50 µM H_2O_2 (A). The *sdrA*::Tn mutant is more susceptible to 500 µM H_2O_2 treatment and showed a significant decrease in fold change compared to the untreated control strain, whereas no difference was observed for the *C. burnetii* NMII or the complemented strain (B). However, both *C. burnetii* NMII and the complemented mutant showed reduced replication after 1mM H_2O_2 treatment (C), suggesting that these strains are also susceptible to higher concentrations of H_2O_2. The treated *sdrA*::Tn mutant was still more susceptible, with significantly reduced replication compared to the treated cultures of *C. burnetii* NMII or the complemented mutant (C). Each experiment was repeated 3 times and significance determined by Student t-test (* $P<0.05$, **, $P<0.01$) relative to untreated or treated group. Error bars represent the standard deviation of mean (n=3). ns= not significant ($P>0.05$*).*

Figure 5. Supplementation of infection medium with 0.025 mM L- ascorbate resulted in a significant increase in replication of the *sdrA*::Tn mutant inside macrophage-like THP-1 cells **A**. Intracellular replication of *C. burnetii* NMII (close circles), *sdrA*::Tn mutant supplemented with L-ascorbate (open squares) and *sdrA*::Tn mutant (closed squares) in THP-1 cells over a seven day period is depicted, with the fold change in genome equivalents relative to day 0 determined at days 1, 3, 5, and 7. Addition of L-ascorbate to the medium resulted in a significant increase in replication of the *sdrA*::Tn mutant (open squares) ($P<0.05$ paired *t*-test) at day 3 and 5 as compared to the untreated *sdrA*::Tn mutant

(closed squares). The experiment was representative of three independent experiments and displayed as mean ± SD. **B**. Immunofluorescent images showing THP-1 cells infected by *C. burnetii* strains for 3 days. The cells were fixed by 4 % PFA and stained with anti-*Coxiella* (red) and anti-LAMP1 (green) antibodies and DNA stained with DAPI (blue). Both *C. burnetii* NMII and *sdrA*::Tn mutant supplemented with L-ascorbate formed much larger CCV containing a large number of bacteria whereas the *sdrA*::Tn mutant in untreated host cells displayed much smaller CCV with fewer *C. burnetii*. A 10 µm scale bar was used for these images that are representative of three independent infections. White asterisks indicate representative CCVs.

Figure 6 Metabolites that are significantly different ($p < 0.05$, BH-adjusted t test) in abundance between *C. burnetii* NMII and *sdrA*::Tn (A) and between the complemented *sdrA* mutant and *sdrA*:Tn (B) in the GC/MS steady state analysis. The metabolites were identified from representative chromatogram by using an Agilent MSD Productivity Chemstation by comparison of retention times and molecular masses with authentic standards. The data were median normalized and log transformed before comparision and are displayed as the average $\log_2$ fold change of six biological replicates.

Figure 7. Labelling of intermediates of central carbon metabolism after one hour of labelling with [^{13}C] glucose, as measured using GC/MS. The percentage of [^{13}C] label incorporated into the total abundance of each metabolite is represented for *C. burnetii* NMII (blue), *sdrA*::Tn (red) and *sdrA*::Tn pFLAG:*sdrA* (green). Error bars represent the standard deviation of the mean from six biological replicates. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ (Student t-test). Abbreviations: G6P, glucose 6-

phosphate; F6P, fructose 6-phosphate; FBP, Fructose 1,6-bisphosphate; Glycerone-P, Glycerone phosphate; Glyceraldehyde-3P, Glyceraldehyde 3-phosphate; 3P-D-glycerate, 3-Phospho-D-glycerate; PEP, phosphoenolpyruvate; Gly, Glycine; L-Ser, L-Serine; L-Ala, L-Alanine; L-Asp, L-Aspartate; TCA, Tricarboxylic Acid Cycle.

Figure 8. **A**. Infection of THP-1 cells results in elevated host cell ROS that is significantly higher in the absence of SdrA. **A.** THP-1 cells were infected for 3 days with *C. burnetii* NMII, the *sdrA*::Tn mutant and *sdrA*::Tn pFLAG:*sdrA*, all expressing mCherry fluorescent protein. THP-1 cells were also seeded in separate 24 well plates as a negative control. The cells were fixed with 4 % PFA for 15 minutes. Host cells were stained with DAPI (blue) and reactive oxygen species were stained with CellROX® Deep Red Reagent (pseudo-coloured green) and the cells were imaged immediately with Nikon A1R confocal microscope. The images are representative of three independent infections. The scale bar represents 10 µm. The boxed area indicates the area from which fluorescent intensity was determined. **B.** The fluorescence intensity produced from CellROX™ channel for both infected and uninfected cells was quantified by imageJ from 50 images per strain per experiment, performed by an investigator blinded to the strains in each sample. The average of each biological replicate (3 in total) is indicated by the individual symbols. The fluorescence signal at CellROX™ channel was higher in infected cells than uninfected cells, demonstrating the oxidative nature of the CCV. The *sdrA*::Tn mutant also showed significantly higher fluorescence intensity, indicating increased oxidative stress in the *sdrA*::Tn mutant when compared to *C. burnetii* NMII and the complemented *sdrA*::Tn mutant (*sdrA*::Tn pFLAG:*sdrA*). Error bars indicate standard deviation from the mean (horizontal line) of three biological replicates, and the symbols are the mean of each biological replicate. Significance was determined using an unpaired two-tailed t-test (*** P<0.001).

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Fluorescent images were taken by confocal microscopes located at the Biological Optical Microscopy Platform, The University of Melbourne. The authors have no conflict of interest to declare.

SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Both 3×FLAG-SdrA (A) and 3×FLAG-G12A (B) were expressed in the *sdrA* mutant following plasmid introduction. A mouse anti-Flag IgG antibody diluted 1/1000 in 1 % (w/v) skim milk in PBS-T was used as a primary antibody and the secondary antibody (anti-mouse IgG HRP) diluted to 1/3000 in 1% (w/v) skim milk in PBS-T was used for screening of clones. Both proteins have a molecular weight of 33 kDa. NTC= Negative control (wild type *C. burnetii* whole cell lysate).

Figure S2. Immunoblotting analysis of recombinant *C. burnetii* SdrA and G12A. The purified recombinant protein was probed with anti-6×His-tag antibody and has a size of approximately 31kDa. NTC= Negative control.

Figure S3. Replication of *C. burnetii* NMII, *sdrA*:Tn mutant and the *sdrA*::Tn complemented mutant (*sdrA*::TnpFLAG:*sdrA*) in ACCM-2 medium over 7 days. Genomic equivalents were determined by *ompA* qPCR at days 0, 1, 3, 5 and 7.

Figure S4. Heat map representing a hierarchical clustering and separation algorithm of *C. burnetii* NMII, *sdrA* mutant and complemented mutant samples analysed by GC/MS. The distinct relative abundances of individual metabolites are represented by different colours. Replicate samples of the *sdrA* transposon mutants were completely separated and clustered together and samples from *C. burnetii* NMII and complemented mutant were overlapped with each other, indicating relatively similar or equivalent abundance. “Class” refers to the strain analysed.

Figure S5. Principal component analysis derived from combined analysis comprising all the groups to highlight their discriminatory potential, with the *sdrA* mutant (green) clustering separately to the complemented mutant (red) and *C. burnetii* NMII (blue). Comp= Complemented *sdrA* mutant, WT= *Coxiella burnetii* NMII, Mutant = *sdrA::Tn* mutant.

Figure S6. Mass isotopologue distribution of TCA cycle intermediates. The +2 labelling pattern suggests that carbon is entering the TCA cycle via acetyl CoA. The data is presented as the mean of six replicates. Comp= Complemented *sdrA* mutant, WT= *Coxiella burnetii* NMII, Mut = *sdrA::Tn* mutant.

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Table 1. Primers used in this study

Name	Sequence (5' to 3')	Annealing temperature (^{o}C)	Purpose
sdrA F1	AAAGTCGACATGCTTTTAAAAGATAAAGTTGTTA	52	Complementation construct
sdrA R1	AAAGTCGACTCATTTAAAAATATCTAATGGAATT	52	Complementation construct
sdrA F2	CGCGGATCCATGCTTTTAAAAGATAAAGTTGTTA	53	Cloning into pQE-30
sdrA R2	AAACTGCAGTCATTTAAAAATATCTAATGGAATT	53	Cloning into pQE-30
ompA F	CAGAGCCGGGAGTCAAGCT	55	Quantification of *Coxiella* genomes (Jaton *et al.*, 2013)
*ompA*9 R	CTGAGTAGGAGATTTGAATCGC	55	Quantification of *Coxiella* genomes (Jaton *et al.*, 2013)
pKM225 F	TGCTCACATGTTCTTTCCTGC	55	Transposon detection
pKM225 R	TGTGATGGCTTCCATGTCG	55	Transposon detection
pJB-Kan:3xFLAG F	GAGCTGTTGACAATTAATCATC	55	Sequencing complementation construct
pJB-Kan:3xFLAG R	GGATTCATCGACTGTGGCCG	55	Sequencing complementation construct
T7	TAATACGACTCACTA	55	Sequencing
SP6	TATTTAGGTGACACT	55	Sequencing
pQE-30 F	CGGATAACAATTTCACACAG	48	Sequencing pQE-30-*SdrA* construct
pQE-30 R	GTTCTGAGGTCATTACTGG	48	Sequencing pQE-30-*SdrA* construct

G12A	F	GTTATCGTAACA**GCT**TCCACGACAGG	55	Site-directed mutagenesis
G12A	R	CCTGTCGTGGAAGCTGTTACGATAAC	55	Site-directed mutagenesis

Underlined represent Gly (**GGT**) mutated to Ala (**GCT**)