

**CHARACTERISING THE ROLE OF METABOLIC ENZYMES IN THE
PATHOGENESIS OF *COXIELLA BURNETII***

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ABSTRACT

Coxiella burnetii is a Gram-negative intracellular pathogen that replicates in a highly acidic and oxidative lysosome-derived vacuole known as the *Coxiella*-containing vacuole (CCV). The relatively recent identification of axenic medium that supports *C. burnetii* replication and the development of improved genetic tools has advanced knowledge of this pathogen and facilitated the identification of virulence factors. A previously conducted transposon mutant library screen using a human cell line identified a number of genes that appeared to be required for intracellular replication, including genes encoding metabolic enzymes such as *nadB* and *sdrA*. To investigate the role of *nadB* and *sdrA* in *C. burnetii* replication, and to link the metabolism of *C. burnetii* with pathogenesis, a number of approaches were used, such as advanced genetic and biochemical techniques, including targeted and untargeted metabolomics. Firstly, each transposon mutant was clonally isolated to avoid wild type contamination. Genetic complementation of both *nadB* and *sdrA* mutants and subsequent intracellular growth assays using both epithelial HeLa cells and macrophage-like THP-1 cells conclusively demonstrated that both *nadB* and *sdrA* are required for efficient *C. burnetii* replication and CCV formation. Analysis of the protein sequence revealed that NadB has a functionally conserved arginine residue at a position of 275 and this arginine was mutated to leucine using site-directed mutagenesis. Purification of both GST-NadB and R275L GST-NadB showed that GST-NadB had L-aspartate oxidase activity, an enzyme catalysing the first reaction of *de novo* NAD synthesis, whereas R275L NadB had lost enzymatic activity. Complementation of *nadB* mutant with a plasmid encoding this inactive R275L NadB was unable to rescue the intracellular replication defect, confirming the requirement of NAD *de novo* synthesis for intracellular replication of *C. burnetii*. Steady state metabolite analysis also showed key changes in the level of abundance of metabolites in the NAD biosynthetic pathway in the *nadB* mutant as compared to parent *C. burnetii* and the complemented *nadB* mutant, demonstrating the role of NadB in *de novo* NAD synthesis. This suggests that this pathway is an ideal target for development of therapeutics, and inhibition of this pathway using a compound non-toxic to mammalian cells reduced *C. burnetii* replication significantly. The role of SdrA in *C. burnetii* metabolism and pathogenesis was also further investigated in this thesis. SdrA is a putative short chain dehydrogenase containing a conserved glycine residue at position 12 that serves as an NADP binding site facilitating NADP(H) regeneration, a key process in resistance to oxidative stress in *C. burnetii*. This Gly residue was replaced by Ala using site directed mutagenesis and purified recombinant 6xHis-G12A was enzymatically inactive when

compared to wild type 6xHis-SdrA that converted NADP^+ to NADP(H) *in vitro*. Complementing the *sdrA* mutant with a plasmid encoding enzymatically inactive 3xFLAG-tagged G12A_SdrA failed to restore intracellular growth, confirming the link between NADP(H) regeneration and *C. burnetii* replication. The *sdrA* mutant showed greater susceptibility to oxidative stress *in vitro* induced by treatment with hydrogen peroxide, whereas both parent *C. burnetii* and the complemented *sdrA* mutant were less sensitive. Supplementation of a commonly available anti-oxidant, L-ascorbate, into the host cell growth medium partially restored the intracellular growth defect of the *sdrA* mutant. Metabolite profiling using GC-MS revealed significant changes in the level of abundance of metabolites of central carbon metabolism in the *sdrA* mutant as compared to parent *C. burnetii* NM II and the complemented mutant. Finally, stable isotope labelling studies using [^{13}C] label glucose showed a change in flux through central carbon metabolic pathways in the *sdrA* mutant demonstrating the presence of oxidative stress. Overall, this thesis has demonstrated the crucial role of NadB and SdrA in *C. burnetii* metabolism and pathogenesis.

DECLARATION

I hereby declare and certify that:

- This thesis is my original work for fulfilment of PhD requirement except where mentioned
- Appropriate acknowledgements have been made in the text of the thesis to all other materials and work used, previously published or written work by another person
- The thesis is less than 100,000 words in length, exclusive of tables, figures, reference and appendices and footnotes

Mebratu A. Bitew

PREFACE

Publications

The following papers have been published from this thesis:

- **Mebratu A. Bitew**, Chen Ai Khoo, Nitika Neha, David P. De Souza, Dedreia Tull, Nadeeka K. Wawegama, Hayley J. Newton, Fiona M. Sansom. (2018). *De novo* NAD synthesis is required for intracellular replication of *Coxiella burnetii*, the causative agent of the neglected zoonotic disease Q fever. *Journal of Biological Chemistry*, 2018, 293:18636-18645. doi:10.1074/jbc.RA118.005190
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Conferences and Symposiums

Research findings from this thesis also involve presentations at domestic and international conferences and symposiums by the student and principal supervisor.

Date	Type of presentation	Title of presentation	Conference venue and location
June 20-24, 2019	Poster presentation	SdrA, an NADP(H)-regenerating enzyme, is crucial for <i>Coxiella burnetii</i> to resist oxidative stress and replicate intracellularly	American Society for Microbiology (ASM Microbe), San Francisco, USA

June 8-11, 2019	Oral presentation	CBU_1276 is Required for Intracellular Replication and Resistance to Oxidative Stress by <i>Coxiella burnetii</i> , the Causative Agent of Q fever	The 30 th meeting of The American Society for Rickettsiology. Santa Fe, USA
Feb, 20-22, 2019	Oral Presentation	The role of metabolic genes in the pathogenesis of <i>Coxiella burnetii</i> , the causative agent of the zoonotic disease Q fever	Lorne Infection and Immunity Conference, Lorne, Australia.
November 29-30	Oral Presentation	<i>De novo</i> NAD synthesis is required for intracellular replication of <i>Coxiella burnetii</i> , the causative agent of the neglected zoonotic disease Q fever	FVAS post-graduate research symposium, Melbourne, Australia
18/10/2018	Oral Presentation	<i>De novo</i> NAD synthesis is required for intracellular replication of <i>Coxiella burnetii</i> , the causative agent of the neglected zoonotic disease Q fever	Victorian Infection and Immunity Network (VIIN), Young Investigator Symposium, Melbourne, Australia
July 1-4, 2018	Oral Presentation	Role of NadB in replication and identification of it as a potential drug target.	Australian Society for Microbiology (ASM 2018), Brisbane, July 2018. Presented by Dr Fiona Sansom (RHD Supervisor)
June 16-19, 2018	Oral Presentation	Investigating the role of metabolic genes in intracellular replication of <i>Coxiella burnetii</i>	The 29 th meeting of The American Society for Rickettsiology. Milwaukee, USA
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25/10/2017	Oral Presentation	Investigating the role of metabolic genes in intracellular replication of <i>Coxiella burnetii</i>	FVAS post-graduate research symposium, Melbourne, Australia
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LIST OF ABBREVIATIONS

ACCM	Acidified Citrate Cysteine Medium
ACP	Acid Phosphatase
AhpC	Alkyl hydroperoxide reductase
ARS	Autonomous Replication Sequence
BDT	Big Dye Terminator Reaction
CCM	Complex <i>Coxiella</i> Medium
CCV	<i>Coxiella</i> -Containing Vacuole
CDC	Center for Disease Control
CTSD	Cathepsin D
DAPI-	4',6-diamidino-2-phenylindole
DHAP	Dihydroxyacetone Phosphate
DMEM	Dulbecco's Modified Eagle's Medium
dNTP	Deoxynucleoside Triphosphates
Dot	Defective organelle trafficking
DTHPA	Dithiohydroxyphthalic Acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
FAD	Flavin Adenine Dinucleotide
FBS	Foetal Bovine Serum
FRD	Fumarate Reductase
GC-MS	Gas Chromatography Mass Spectrometry
Grxs	Glutaredoxins
Icm	Intracellular multiplication

iNOS	inducible Nitric Oxide Synthase
LAMP1	Lysosome-Associated Membrane Protein 1
LASPO	L-aspartate Oxidase
LB	Lysogeny Broth
LC3	Light-Chain 3
LC-MS	Liquid Chromatography Mass Spectrometry
LCV	Large Cell Variant
LPS	lipopolysaccharide
MOI	Multiplicity of Infection
Msrs	Methionine sulfoxide reductases
M β -CD	Methyl- β -Cyclodextrin
NA	Nicotinic Acid
NaAD	Nicotinic Acid Adenine Dinucleotide
NAD	Nicotinamide Adenine Dinucleotide
NaMN	Nicotinic Acid Mononucleotide
Nm	Nicotinamide
NMII	Nine Mile phase II
NMN	Nicotinamide Mononucleotide
PCR	Polymerase Chain Reaction
Phox	Phagocyte oxidase
PMNs	Polymorphonuclear Monocytes
PTS	Phosphotransferase System
Qa	Quinolate
RNS	Reactive Nitrogen Species

ROS	Reactive Oxygen Species
RPM	Revolution per Minute
RPMI	Roswell Park Memorial Institute Media
SCV	Small Cell Variant
SDH	Succinate Dehydrogenase
SDRs	Short-Chain Dehydrogenases
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SOD	Superoxide Dismutase
T1SS	Type I Secretion System
T2SS	Type 2 Secretion System
T4SS	Type 4 Secretion System
TCA	Tricarboxylic Acid Cycle
TEMED	Tetramethylethylenediamine
Trxs	Thioredoxins

Chapter 1. Introduction

1.1. Historical background

Coxiella burnetii is a Gram negative, intracellular bacterium that causes the neglected zoonotic disease Q fever. “Q” stands for “query”, as the causative agent of the first outbreak of this disease, in Queensland, Australia, in 1935, was initially unknown (1). Nine patients working in an abattoir in Brisbane developed a febrile illness of unknown cause. Edward Derrick, the physician in charge of investigating the outbreak, infected guinea pigs with blood from infected patients, but could not detect the causative agent (1). He then supplied infected tissue from the guinea pigs to Frank Macfarlane Burnet and Mavis Freeman who infected guinea pigs and observed filterable organisms of a “rickettsial nature” in splenic smears (2). However these infected tissues did not react with serum from patients infected with typhus rickettsia. At the same time an unfamiliar infectious agent was identified in *Dermacentor andersoni* ticks collected in the Rocky Mountains in Hamilton, Montana, USA, near Nine Mile Creek (3). The agent passed through filters and was infectious to guinea pigs, and had a Gram-negative rickettsia-like appearance (4). The Nine Mile agent was unable to grow in host-cell free medium, but was filterable and could be serially passaged in guinea pigs. Based on these findings it was concluded at the time that the agent was not a “true” bacterium (3).

The association between the Nine Mile and Q fever agents was discovered when laboratory workers were accidentally infected by the Nine Mile agent, as the clinical signs and symptoms observed were typical of Q fever (5). Dyer (5) confirmed that there was cross protection between Q fever and Nine Mile agents and that the two agents were likely the same pathogens. The agent was categorized under a new genus in family Rickettsiaceae as the organism didn’t show typical feature of rickettsia, and named as the genus “*Coxiella*” and the species “*burnetii*” in honour of Cox and Burnet, respectively. More recently, based on the sequence of its 16S rRNA gene, *C. burnetii* was reclassified from the order Rickettsiales to the phylum Proteobacteria, class gamma Proteobacteria, order Legionellales, and family Coxiellaceae (6,7).

1.2. Clinical presentation and epidemiology of Q fever

A zoonotic disease, Q fever is a disease mainly transmitted by inhalation of soil or animal products contaminated by *C. burnetii*. Domestic ruminants are a key reservoir of infection for this pathogen, but it may also infect a wide range of wild species and arthropods (8). The

disease causes high morbidity, but rarely causes mortality if treated. The organism has a low infectious dose and can survive for long periods in the environment, and thus is categorized as a class B select agent by the US CDC, because it may serve as a biological weapon (9). In the acute form of the disease clinical signs may develop 2-6 weeks post infection, although in more than 50% of cases the infection does not result in any clinical signs. Clinical signs can include severe headache, pneumonia, hepatitis, myalgia, malaise, high fever, and the acute phase lasts for 1-2 weeks (10). The acute phase of the disease can currently be fully treated with antibiotics such as tetracycline and third-generation cephalosporins (11,12). However, it is estimated that 1-2% of acute Q fever develops into a chronic infection which is characterised by endocarditis, hepatitis or the recently described post-Q fever fatigue syndrome (13,14).

To date, the highest recorded outbreak of Q fever occurred in the Netherlands that cost over 307 million Euro and required the culling of tens of thousands of animals to control (15). More than 4000 human cases of Q fever infections were reported in the Netherlands between 2007 and 2010. The southern part of the country was the most common site of cases as most dairy goat farms were located in this region, with a number of herds in close proximity to urban centres. This type of outbreak represents a significant threat to public health. The number of Q fever cases reported in the Netherlands has reduced following implementation of control measures including immunisation of small ruminants, culling of infected herds, and improved hygiene procedures (16).

1.3. Intracellular lifestyle of *C. burnetii*

C. burnetii grows inside the host cell within a vacuole derived from the host phagolysosome, known as the *Coxiella*-Containing Vacuole (CCV). In natural infection, *C. burnetii* has an affinity for alveolar macrophages, but it can also replicate in a wide range of both phagocytic and non-phagocytic cells (17). There exists two forms of *C. burnetii*, the metabolically active and replicating large cell variant (LCV) and the non-replicative small cell variant (SCV) (18). The SCV is characterised by resistance to physical and chemical factors and may survive in the environment for many months (18). The shift from SCV to LCV occurs after entry into the host cell. Acidification of the phagolysosome, more usually a mechanism for the host cell to kill invading bacteria, is actually required for *C. burnetii* to become metabolically active and begin replicating (18,19). *C. burnetii* enters the host cell passively via classical actin-dependent phagocytosis and resides in a tightfitting nascent phagosome during the first 4–6 hours post-infection (20). Once the bacterium is internalised into a host cell, there is recruitment of small

GTPases such as Rab5 and Rab7, which indicates the CCV is trafficked through the classical endosomal pathways (21) (Figure 1) leading to maturation of the CCV, which eventually occupies the majority of the host cytoplasm (17,22) (Figure 1). This maturation and expansion of the CCV requires functional Rab5 and Rab7 (21). However, inhibition of Rab5 alone results in blockage of entrance of *C. burnetii* into host cells (21). Moreover, active *C. burnetii* protein synthesis is also vital for the development of large CCV, which is essential for production and secretion of effector proteins from CCV membrane into the cytoplasm (23,24). The lysosomal hydrolases are also required for normal CCV biogenesis as defects in CCV formation occurred in HeLa cells lacking Golgi, N acetylglucosamine-1-phosphotransferase (GNPT) activity (25). However, the replication of *C. burnetii* in HeLa cells that lacks lysosomal hydrolases in endocytic compartments (GNPTAB^{-/-} 32) was not impaired demonstrating the lysosomal hydrolases are not required for growth and survival in CCV(25).

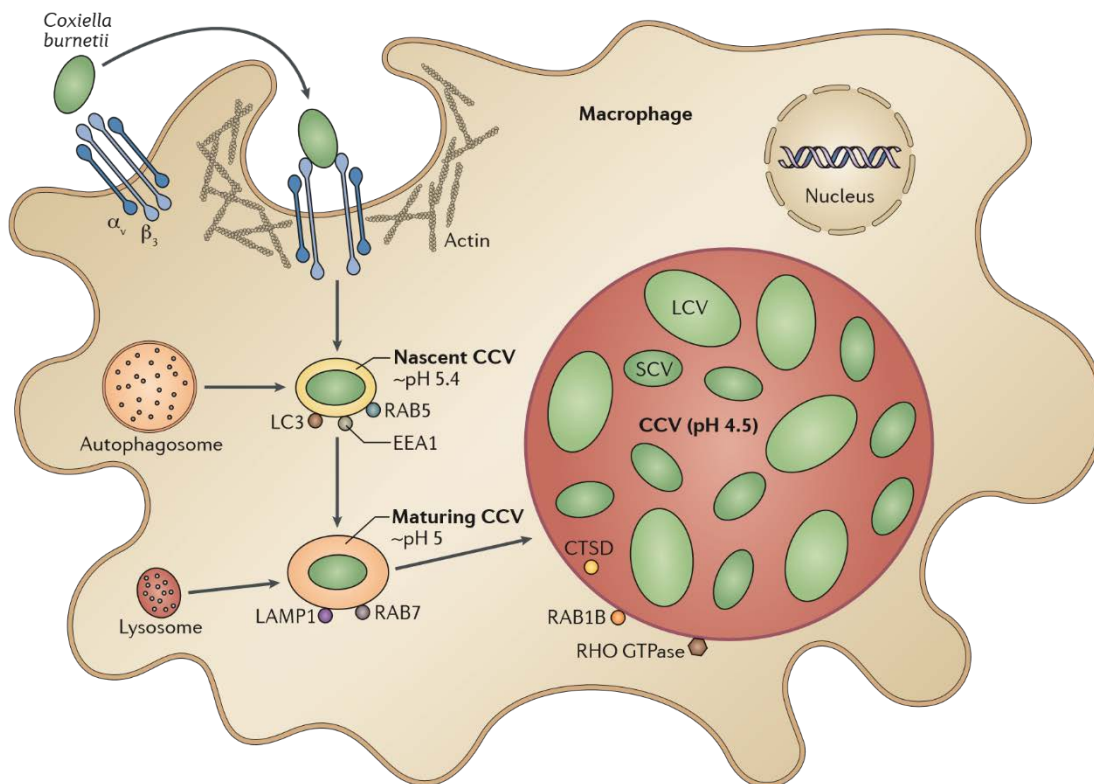


Figure 1. The intracellular trafficking process of *Coxiella burnetii* (26). The process starts with the binding of *C. burnetii* to macrophages via $\alpha\text{v}\beta 3$ integrin which initiates bacterial phagocytosis using an actin-dependent mechanism. Five minutes after internalization, the nascent *Coxiella*-containing vacuole (CCV) will obtain RAB5 and EEA1 and creates an acidic environment with a pH of 5.4 which is a typical feature of phagosomal development. As compared to phagosomes, the CCV also obtains microtubule-associated protein light-chain 3 (LC3), a process that is dependent on bacterial protein synthesis. 40 to 60 minutes post-internalisation the fusion and fission of early and late endosomes leads to maturation of nascent CCV which is characterised by disappearance of RAB5 and EEA1 and acquires a new GTPase RAB7 and lysosome-associated membrane glycoprotein 1 (LAMP1) and acidifies to a pH of 5. Two hours after internalization the CCV acquires lysosomal enzymes such as cathepsin D (CTSD) and the pH of the CCV becomes 4.5. The CCV expands and occupies the major part of host cell cytoplasm 8 hours and 2 days post internalization. Bacterial protein synthesis plays a crucial role on this process and is characterised by the recruitment of RHO GTPase and RAB1B to the CCV membrane. RHO GTPase helps to maintain the large vacuole, whereas RAB1B involves in facilitating acquisition of additional membranes for the formation of this spacious CCV.

1.4. Known virulence factors of *C. burnetii*

Two relatively recent breakthroughs in *C. burnetii* research, namely the discovery of host cell free medium capable of supporting *C. burnetii* replication (27) and improvements in the tools for genetic manipulation (28), have significantly advanced the understanding of *Coxiella* pathogenesis. In addition to the known virulence factor lipopolysaccharide (LPS) (29,30), the Type 4 secretion system (T4SS) (31), the Dot/Icm, was recently shown to be essential for intracellular replication, and a large number of effector proteins of this T4SS have now been identified and characterised.

1.4.1. LPS

The first virulence factor to be identified for *C. burnetii* was LPS, based on the observation that serial passages in a host led to a switch from a smooth to a rough phenotype, and this rough phenotype lost virulence and pathogenicity (32). Phase I *C. burnetii* is highly pathogenic due to expression of full length lipopolysaccharide antigen (LPS) (the smooth phenotype) (33).

After subsequent *in vitro* passage of phase 1 organisms in synthetic medium acidified citrate cysteine medium-2 (ACCM-2), spontaneous mutations lead to phase II *Coxiella* that express a truncated LPS (the rough phenotype) that are highly attenuated within *in vivo* models of infection (30). Genome sequencing of strains identified several genes required for biosynthesis of LPS and genetic mutation of these genes and deletion of a single amino acid resulted in production of truncated phase II LPS (30). The *C. burnetii* Nine Mile strain RSA439 is a locked phase II variant and is avirulent, because of a chromosomal deletion that eliminates synthesis of O-antigen polysaccharide (30). It has been shown by others that the characteristics of this Nine Mile II strain in tissue culture models of infection are the same as phase I *C. burnetii*, and hence this strain usually serves as a safer model in laboratories to study the molecular pathogenesis of *C. burnetii* (34).

1.4.2. The Dot/Icm Type 4 secretion system

Bioinformatic analysis of the *C. burnetii* genome revealed three secretion systems, namely, type I secretion system (T1SS) (35), a type 2 secretion system (T2SS)-related pilus biogenesis machinery (36) and a type 4 secretion system (T4SS) (37). The role of the T1SS and T2SS in the molecular pathogenesis of *C. burnetii* is currently unknown. The closely related bacterium *Legionella pneumophila* has a similar T4SS that is essential for replication inside cells (38,39). This system was first characterised in this bacterium simultaneously by two separate research groups, leading to the dual naming of the system as the Dot/Icm (defective organelle trafficking (Dot) and intracellular multiplication (Icm)) (40). In *L. pneumophila*, the Dot/Icm translocates a large number of effector proteins from the bacterium across the membrane surrounding the LCV into the host cell. In *C. burnetii*, the Dot/Icm T4SS is also essential for intracellular replication (31).

However, in contrast to *L. pneumophila* where there appears to be redundancy in the function of many effector proteins, in *C. burnetii* a number of effectors appear important for intracellular replication. Recently characterised Dot/Icm T4SS effector proteins include Cig57, CoxCC8 and CBU1754, which are all individually required for efficient intracellular growth of *C. burnetii* (41). In the same study Cig2 was also identified as an effector protein essential for homotypic fusion of CCV (41). Additional effectors include CvpA, which interacts with the clathrin adaptor protein (AP-2) to modulate the association of *C. burnetii* with the clathrin trafficking pathway (42), and CvpB, which modulates phosphoinositide metabolism for optimal vacuole development (43).

1.5. Host cell free culture of *C. burnetii*

The discovery of host cell free medium for propagation of *C. burnetii* was a crucial step in allowing more detailed and faster research into the molecular pathogenesis of the organism. This development was made after investigating the potential for acid activation buffer to support *C. burnetii* metabolic activity (44). The second discovery came from the finding that there is correlation between micro-aerophilic respiration and terminal oxidases (27). Substrate oxidation by *C. burnetii* increases with decreasing oxygen availability and *C. burnetii* can respire under low oxygen conditions due to the fact that this pathogen encodes terminal oxidases associated with aerobic (cytochrome *o*) and microaerobic (cytochrome *bd*) metabolism (27). This investigation led researchers to discover that a low oxygen level, 2.5%, is ideal for growth and metabolism of *C. burnetii* (27). Based on these two findings acidified citrate cysteine medium (ACCM) was developed which enables a ~3 log increase in the number of genome equivalents after 6 days of growth (27). Genomic equivalent is defined as a copy of *dotA/ompA* as being equivalent to one bacterial cell, based on the assumption that there is a single copy in each cell (45). A second-generation medium, acidified citrate cysteine medium-2 (ACCM-2), was developed aimed at increasing replication and viability (46,47). This medium also allowed sufficient growth to facilitate the genetic manipulation of the organism and selection of transformants using antibiotics (48-50). Metabolic reconstruction of *C. burnetii* indicated that, the bacterium cannot synthesize all amino acids and must get some from the host (51). Recently, a new nutritionally defined medium with known amino acid concentrations, termed ACCM-D, was developed to support greater replication and longer logarithmic growth (51).

1.6 Genetic manipulation of *C. burnetii*

The inability to grow *C. burnetii* outside host cells restricted genetic manipulation of the organism and hampered investigation into the molecular determinants of virulence. Although bioinformatic analysis could identify genes encoding likely *C. burnetii* virulence factors (35,52), the approaches for gene inactivation and transcomplementation were extremely difficult, so applying molecular Koch's postulates (53) was not practical. However, the role of *C. burnetii* genes and their regulation were investigated using heterologous expression in alternative hosts (54-56). For example *C. burnetii* was genetically transformed for the first time by Suhan et al. (57) in 1996. This transformation was achieved by using a *C. burnetii* - *E. coli*

shuttle vector, pSKO(+)1000, a construct produced by cloning a 5.8 kb *C. burnetii* autonomous replication sequence, *ars*, into the *E. coli* cloning vector pBluescript which has a β -lactamase gene (*blaM*) conferring ampicillin resistance. Similar to previous studies in chlamydia (58), this study was a major milestone and breakthrough for transformation of *C. burnetii* by presenting these important points: (1) DNA is introduced into *C. burnetii* by electroporation, (2) ampicillin was used as a potential selectable marker, (3) the occurrence of homologous recombination, and (4) pSKO(+)1000 served as a shuttle vector.

A major advance occurred in 2009 when Beare et al. (59) transformed host cell-free cultivated *C. burnetii* using the mariner-based Himar1 transposon (Tn). Isolation of individual colonies on solid ACCM-2 agarose plates (46) facilitated the generation of transposon mutant libraries using Himar1-based transposon system (59). A site-specific Tn7 transposition system was also developed as an additional option for transformation with plasmids (28). Recently, a directed mutagenesis approach has been established that facilitates investigation of virulence candidate genes in *C. burnetii* mutants by targeted gene deletion and inactivation (48).

1.7. Metabolism of *C. burnetii*

Complete genome sequencing of *C. burnetii* strains has allowed detailed investigation of *C. burnetii*'s metabolic potential (35,52). Furthermore, as examination of *C. burnetii* ORFs revealed, this organism encodes complete central metabolic pathways, such as glycolysis, the tricarboxylic acid cycle (TCA), and pathways for nucleic acid biosynthesis. A study of the organism's metabolic abilities in buffers indicated extensive activity of central metabolic pathways (e.g., glycolysis and RNA synthesis) in bacterial extracts, but not intact cells (60).

Early biochemical investigations demonstrated that *C. burnetii* can catabolize glucose (44). However, this pathogen lacks the first enzyme of glycolysis, the glucokinase or hexokinase that is essential for conversion of glucose to glucose-6-phosphate. However, *C. burnetii* may use alternative mechanisms to facilitate the flux of carbon from glucose into G6P via sugar phosphotransferase system (PTS, CBU1550) and the histidine phosphocarrier protein (HPr, CBU0743) (61,62). Phosphorylation of glucose by *C. burnetii* lysates in the presence of carbamoyl phosphate suggests that an alternative carbamoyl phosphate-dependent mechanism is present, but the gene(s) encoding the enzyme(s) responsible for this process remain unknown (63). Previous studies indicated that *C. burnetii* possesses a complete TCA cycle and make use of glutamate, succinate, and pyruvate (44). Amino acid biosynthetic pathway reconstruction showed that *C. burnetii* cannot synthesize all amino acids and is auxotrophic for some amino

acids. Thus, *C. burnetii* must acquire these amino acids through transport of free amino acids or peptides from the CCV lumen (64). A more recent study developed a nutritionally defined medium with known amino acid concentrations, termed ACCM-D that allowed determination of *C. burnetii* amino acid auxotrophies (51). A defect in growth in amino acid-deficient formulations of ACCM-D demonstrated that *C. burnetii* auxotrophy for 11 amino acids, including arginine (51). Glucose labelling at low levels into some of these amino acids, suggest that there is a low level of synthesis for several but that *C. burnetii* still needs them for growth, presumably as rate of synthesis is not adequate (61).

Genome analysis reveals that *C. burnetii* does not encode the glyoxylate bypass enzymes isocitrate lyase and malate synthase, suggesting it cannot utilize fatty acids as a sole source of carbon, in contrast to another intracellular bacterium *Mycobacterium tuberculosis*, which is dependent on fatty acids as a source of carbon for its intracellular growth (65). *C. burnetii* encodes a complete non-oxidative part of the pentose phosphate pathway which enables production of pentose sugars for nucleic acid synthesis, but lacks glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase and thus does not encode the oxidative branch of this pathway. The bacterium therefore must possess an alternative mechanism for NADP(H) regeneration, which is an essential source of reducing equivalents for biosynthetic reactions and antioxidant mechanisms (66). Generally, biochemical and genomic data indicate that *C. burnetii* utilizes energy derived from oxidation of glycolytic and TCA cycle intermediates, and substrates that tunnel directly into these pathways.

The genome sequence of other obligate intracellular pathogenic bacteria also showed their metabolic potentials and the putative central metabolic pathways. Due to their adaptation to an intracellular lifestyle, these obligate intracellular bacteria, possess a significantly reduced metabolic capabilities and are mainly dependent on a wide range of host-derived metabolic precursors and cofactors (67). For example, Chlamydia encode the enzymatic capacity for metabolism of glucose-6-phosphate to pyruvate via glycolysis which enables them to generate ATP via substrate-level phosphorylation ATP by the help of pyruvate kinase and phosphoglycerate kinase enzymes (67). The TCA cycle is incomplete in Chlamydia due to lack of enzymes such as isocitrate dehydrogenase (Icd), aconitase (Acn) and citrate synthase (GltA). However, Chlamydia possess a complete pentose phosphate pathway essential for regeneration of NADPH and synthesis of pentose-phosphates, a complete pathway for gluconeogenesis, and the capacity to synthesize and degrade the energy storage compound glycogen(67).

For example, *M. tuberculosis*, possess several *fad* genes encoding the enzymes for fatty acid uptake and degradation by β -oxidation, genes essential for cholesterol uptake and catabolism,

genes required for the glyoxylate shunt, including two isogenes encoding isocitrate lyases and a gene encoding malate synthetase (68) which enables *M. tuberculosis* to efficiently use fatty acids and cholesterol as carbon sources (69).

The metabolism of pathogenic bacteria depends on carbon sources for energy production and generation of catabolic intermediates required for synthesis of anabolic monomers used in the reactions leading to macromolecule generation such as proteins, nucleic acids and complex cell envelope components. Several uptake systems and pathways are involved in the transport of nutrients and the genes involved in these pathways are regulated by both global and specific transcriptional factors that may sense key metabolites produced in the central carbon metabolic pathways (70). Bacterial pathogens reprogram their metabolic network to establish infection, by maintaining biosynthetic processes with adequate ATP synthesis to support both growth and survival (70,71). These altered metabolic pathways are crucial for bacterial survival *in vivo* and are potential targets for development of antimicrobial agents. Due to lack of selectivity and the conserved nature of most metabolic enzymes between bacteria and human, central metabolism has been considered an unattractive target for antimicrobial development historically. A detailed understanding of how cells regulate the flux of nutrients into pathways that are essential for biosynthesis and energy metabolism under relevant growth conditions may lead to success in targeting bacterial central metabolism (72).

1.8. Nicotinamide adenine dinucleotide (NAD) biosynthesis

Nicotinamide adenine dinucleotide (NAD) is a multifunctional coenzyme found in all living cells (73). It is a dinucleotide compound consisting of two nucleotides joined together through their phosphate groups. The first nucleotide is made up of adenine base and the other is nicotinamide (74). This molecule is involved in redox metabolism carrying electrons from one reaction to another.

Although a number of environmental factors including ionic strength, osmotic pressure, electronic pulse, temperature and pH affect the growth and metabolism of microorganisms (75), redox metabolism has received relatively little attention over the last several years. In order to adapt to these various environmental conditions microbes have developed an enormous variety of electron transport chains (76). In microbial electron transport chains electrons are transferred from a low potential electron donor to an acceptor with more positive redox potential by redox reactions (76). Important systems catalysing these redox reactions include primary dehydrogenases that supply high energetic electrons from a donor such as NADH and

usually couple electron transport to H^+ or Na^+ transport across the membrane (76). These redox reactions need to be balanced to maintain the growth and survival of bacterial pathogens (77). Oblinger and Kraft (78) showed that oxidation-reduction balance is vital for growth of *Salmonella spp.* and *Pseudomonas fluorescens*. This is also true for the Gram-positive pathogenic bacterium *Clostridium perfringens* that requires a balanced redox metabolism for growth (79) and also for *Clostridium botulinum* (80). Results from previously conducted research provided an initial evidence that redox metabolism is important during intracellular replication of *C. burnetii* (31). The redox balance is also vital for maintaining cellular homeostasis and is critical for the proper functioning of the cells and the immune system, but the imbalance of the redox molecules will result in oxidative stress (81). Therefore, the coenzyme exists in two forms in cells: NAD^+ is the oxidizing agent which accepts electrons from other molecules and becomes reduced. From this reaction NADH is formed which serves as a reducing agent to donate electrons. This transfer of electrons is the primary role played by NAD. However, it is also used as a signalling molecule in a variety of cellular processes, such as a co-substrate of enzymes that add or remove chemical groups from proteins in posttranslational modifications (82). Because of these crucial functions, the enzymes involved in NAD metabolism are potential targets for discovery of therapeutics.

Some genes in the NAD biosynthetic pathway are also essential for *M. tuberculosis* viability (83). However, research suggests that either *de novo* synthesis or the recycling pathway is independently sufficient to support the *in vivo* replication of *M. tuberculosis*. The *pncB1::aph/pncB2::hyg* mutants were able to infect and replicate similar to the wild type in host cells suggesting that NAD biosynthesis did not result in a significant loss of bacterial virulence (84). *pncB1::aph/pncB2::hyg* are the knock-out mutants of *M. tuberculosis pncB1* and *pncB2* (two putative *pncB* homologs) and are functional nicotinate phosphoribosyltransferases involved in salvage synthesis (84). Knockdown of both NadD and NadE in *Mycobacterium smegmatis* results in changes in the level of nicotinic acid mononucleotide (NaMN) and nicotinic acid adenine dinucleotide (NaAD) intermediates (substrates of NadD and NadE, respectively) and depletion of the NAD(H) pool and showed strong bactericidal effects (83).

On the other hand, the Gram-negative bacterium *Shigella flexneri* does not possess a functional NAD *de novo* biosynthesis pathway due to mutation of *nadA* and *nadB* genes (85). In *C. burnetii*, in contrast to the situation in *M. tuberculosis*, recent work by Newton et al. (31) suggested that the NAD biosynthetic pathway may be required for efficient intracellular replication, as a number of mutants containing transposon insertions into *nadB* were attenuated

in a screen for growth inside HeLa cells . Given the highly oxidative environment of the phagolysosome, it is expected that genes associated with redox balance are important to *C. burnetii* intracellular survival and replication.

1.8.1. *De novo* NAD biosynthesis pathway

Most prokaryotes such as *E. coli* and *C. burnetii* possess the canonical *de novo* NAD biosynthesis pathway that begins with conversion of aspartate (Asp) to iminoaspartate via NadB, an L-aspartate oxidase. The second enzyme NadA, a quinolinate synthase, catalyses the formation of quinolinate from iminoaspartate and this quinolinate is subsequently converted to nicotinic acid mononucleotide (NaMN) via NadC, a quinolinate phosphoribosyl transferase, resulting in the synthesis of NAD (Figure 2 and 3). In contrast, most eukaryotes including humans have evolved to use the five-step pathway from tryptophan (Trp) (Figure 2). Some prokaryotes such as *Haemophilus influenza* lack genes encoding the *de novo* pathway (86) and a minority of prokaryotes, such as the soil bacterium *Cytophaga hutchinsonii*, encode the *de novo* pathway from Trp (87). Furthermore, some eukaryotes such as the fungus *Candida glabrata* lack the genes for a *de novo* pathway (88), and some plant species, such as *Arabidopsis thaliana*, carry the genes for a *de novo* pathway from Asp (89).

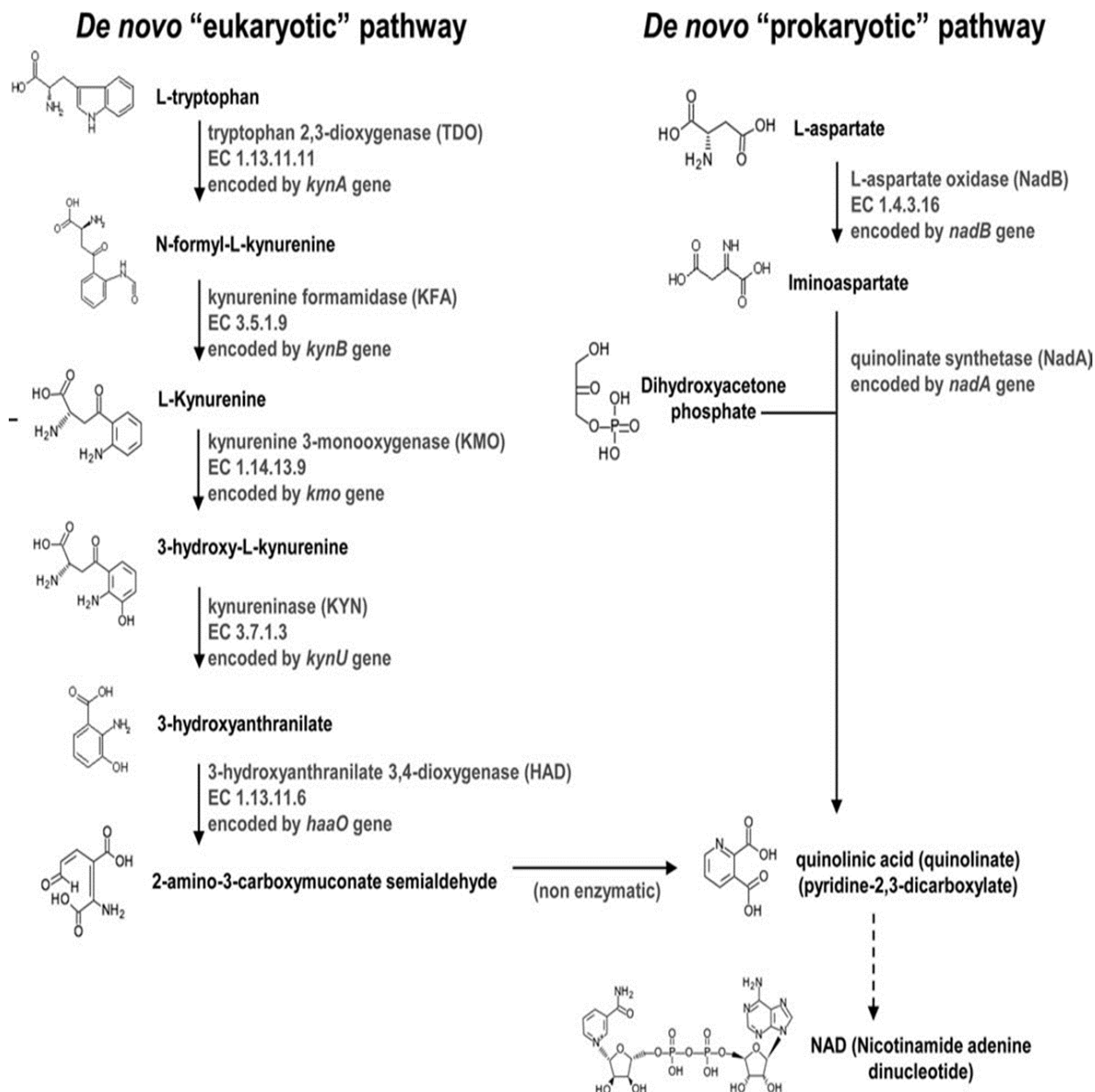


Figure 2: Schematic diagram showing the two major NAD *de novo* biosynthesis pathways (90). Both pathways synthesize quinolinic acid, either through a five-step eukaryotic route from Trp (most eukaryotes) or a two-step pathway from Asp (most prokaryotes).

they are produced inside the cells by breakdown of cellular NAD^+ . There are also other reports that eukaryotic cells can take up extracellular NAD^+ from their surroundings (92) and both nicotinamide and nicotinamide riboside can be absorbed from the gut in mice and humans (93). The salvage pathways used in humans and other eukaryotes are different from those used in prokaryotes (94). Bacteria such as *Bordetella* species (95) and *Streptococcus pyogenes* (96) use salvage pathway genes *pncA* and *pncB*, for utilisation of nicotinamide or nicotinic acid, respectively, which enables them to synthesize NAD. These pathways utilise NAD precursors or breakdown products which are taken into the cell or produced inside the cells and fed into the NAD synthesis pathway, bypassing early *de novo* steps. These salvage pathways are dispensable for some model prokaryotes including *E. coli* and *S. cerevisiae* (97). The intracellular pathogen *Chlamydia trachomatis* lacks genes involved in biosynthesis or salvage and is entirely dependent on direct uptake of NAD^+ and NADP^+ from its host (98). In most pathogenic bacteria the pathways downstream of *de novo* biosynthesis and the salvage pathways are common (Figure 3). These include conversion of nicotinate mononucleotide (NaMN) to nicotinic acid adenine dinucleotide (NaAD) which is catalysed by nicotinate mononucleotide adenylyltransferase (NadD) and conversion of nicotinic acid adenine dinucleotide (NaAD) to NAD facilitated by NAD synthetase (NadE). In *C. burnetii*, genome analysis suggest the Pnc salvage pathway is absent. Instead there exists *cbu_1035* encoding a putative nicotinate phosphoribosyltransferase, which may catalyse the conversion of scavenged nicotinic acid to NaMN (62).

1.8.3. The function of NAD

The compounds NAD, NADP, NADP(H), and NADH serve as hydride acceptors and donors in many cellular and biological reactions (97). The classical function of NAD^+ in metabolism is its role in electron transfer from one molecule to another. These kind of reactions are catalysed by a group of enzymes called oxidoreductases. The redox reactions catalysed by this type of enzymes are crucial in all type of metabolism, but more importantly these reactions result in the release of energy from nutrients. The energy released from oxidation of reduced compounds such as fatty acids and glucose is transferred to NAD^+ by reduction to NADH, as part of beta oxidation, glycolysis, and the TCA cycle (99). In eukaryotic cells the electrons generated in the cytoplasm and carried by the NADH are transferred to mitochondrion (to reduce mitochondrial NAD^+) via mitochondrial shuttles including malate-aspartate shuttle (99). The NADH in mitochondria is then oxidized by the electron transport chain resulting in

pumping of protons across a membrane and produce ATP via oxidative phosphorylation (100). Since reduced and oxidized forms of NAD are consumed in these linked reactions, significant levels of NAD^+ and NADH with a high NAD^+/NADH ratio will be maintained by the cells allowing this coenzyme to serve as both reducing and oxidizing agent (73).

In addition to its vital role in redox metabolism, NAD also serves as a substrate for Poly (ADP-ribose) polymerases (PARPs), bacterial DNA ligases, and Sir2- and CobB-related protein lysine deacetylases termed sirtuins (97). Poly (ADP-ribose) polymerases (PARPs) are a group of enzymes which catalyses the transfer of ADP-ribose to target proteins (101). PARPs are involved in a variety of biological and cellular reactions such as transcription, replication, recombination, modulation of chromatin structure and DNA repair (101). For example, DNA strand breaks or metabolic insults initiates overactivity of PARP-1 leading to NAD^+ exhaustion and bioenergetic failure (102) which induce caspase-independent apoptosis (103). The sirtuins are a class of proteins that act predominantly as nicotinamide adenine dinucleotide (NAD) - dependent deacetylases activity or possess mono-ADP-ribosyltransferase activity (104,105). The deacetylation reactions driven by sirtuins link lysine deacetylation to NAD hydrolysis. The dependence of sirtuins on NAD links their enzymatic activity directly to the energy status of the cell via the cellular NAD:NADH ratio, the absolute levels of NAD, NADH or nicotinamide or a combination of these variables. Moreover, metazoans and other multicellular eukaryotes have developed NAD-utilising activities, including cADP-ribose synthetases and poly(ADP-ribose) transferases (73).

1.8.4. Inhibitors of NAD biosynthesis

NAD^+ is an essential molecule in cell metabolism and the enzymes involved in bacterial *de novo* NAD^+ biosynthesis are possible targets for novel therapeutic agents against pathogenic bacteria, particularly due to the differences between pathways in humans and most prokaryotes (106). A study carried out by Gerdes et al. (98) also suggested that as NadA and NadB are absent in mammals, they are considered to be ideal targets for the development of novel prophylactic and therapeutic agents. A number of potential inhibitors has been studied previously. For example, mesotartrate can completely inhibit L-aspartate oxidase activity (107). The L-aspartate oxidase is a flavoenzyme which contains one mole of covalently bound flavin adenine dinucleotide (FAD) required for its enzymatic activity as a cofactor and is also competitively inhibited by NAD^+ with respect to FAD (107).

A combined *in vitro* and *in vivo* inhibition studies carried out on *E. coli* showed that quinolinate synthase (NadA) activity is inhibited by 4,5- dithiohydroxyphthalic acid (DTHPA) which leads to loss of quinolinate formation (108). The *E. coli* growth was affected by 10 μ M DTHPA and totally inhibited with 20 μ M DTHPA. A structurally similar compound, phthalate, is a competitive inhibitor of NadC (quinolinate phosphoribosyl transferase), which prevents conversion of quinolinate to nicotinic acid mononucleotide thereby leading to inhibition of NAD synthesis (109). A study conducted on *M. tuberculosis* also showed that quinolinate phosphoribosyl transferase is inhibited by an anti-tuberculosis drug pyrazinamide (which is itself a structural analogue of quinolinate), which in turn leads to absence of NAD synthesis (110). Moreover, a number of inhibitors were also screened and identified for nicotinate mononucleotide adenylyltransferase (NadD), an enzyme facilitating the conversion of nicotinate mononucleotide (NaMN) to nicotinic acid adenine dinucleotide (NaAD) (111).

There are marked differences in early steps in NAD biogenesis and salvage pathway between species, but the enzymes catalysing the downstream pathway such as conversion of nicotinic acid mononucleotide (NaMN) to NAD exist in nearly all analysed bacterial genomes (112). Therefore all these downstream enzymes of the pathway such as NaMN adenylyltransferase (EC 2.7.7.18) NadD, NAD synthetase (EC 6.3.1.5) NadE and the NAD kinase (EC 2.7.1.23) NadF are attractive antibacterial targets. NadD is ideal target for the development of specific inhibitors due to broad conservation and structural and functional distinctions from its human counterpart (98,113) NadD has a strong substrate specificity and preference for NaMN over its amidated analog, NMN (114) whereas functionally equivalent human enzyme (hsNMNAT) has similar catalytic efficiency and substrate specificity for both NaMN or NMN (115,116). hsNMNAT and NadD have the lowest sequence similarity (98). There are also major differences between their catalytic site conformations between bacterial NadD and hsNMNAT which is revealed by comparative analysis of their 3D structures (113). This could be the reason for their substrate specificity and allow them for selective targeting.

1.8.5. *L*-aspartate oxidase

L-aspartate oxidase (LASPO) is a flavo enzyme which catalyses the first reaction of bacterial NAD *de novo* biosynthesis pathway. The enzyme facilitates oxidation of L-aspartate to iminoaspartate and contains 1 mol of noncovalently bound FAD as a prosthetic group (106). In addition to its vital role in synthesis of NAD, LASPO is also a member of the FAD-dependent oxidoreductase group which contains the flavoprotein subunits of fumarate

reductase (FRD) and succinate dehydrogenase (SDH). These group of enzymes share sequence similarity (30%) and functional properties such as the capacity to catalyse fumarate reduction and oxidise substrates bearing two carboxylate groups (106). The *C. burnetii* LASPO is comprised of 536 amino acid residues whereas *E. coli* LASPO consists of 540 amino acid residues, and most of the key residues involved in substrate binding and catalysis are conserved. Many organisms possess a *nadB* gene, but the protein has only been purified from some organisms such as *Escherichia coli*, *Pyrococcus horikoshii* and *Sulfolobus tokadaii*, and characterized from a biochemical and structural point of view only for *E. coli* and *S. tokadaii* (117-119). These enzymes possess some distinguishing features from other flavo-oxidases: (a) *in vitro*, this enzymes utilizes oxygen, fumarate, cytochrome c and quinones as electron acceptors for FAD oxidation (117) suggesting that it is involved in NAD biosynthesis in anaerobic as well as aerobic conditions; and (b) the primary and tertiary structures are not exactly the same as other flavo-oxidases, but to those of the flavoprotein subunit of the succinate dehydrogenase/fumarate reductase class of enzymes (120,121).

1.9. Short chain dehydrogenase/reductase oxidoreductase

In addition to identifying *nadB*, which encodes the L-aspartate oxidase required for *de novo* NAD synthesis, *cbu_1276* was also identified in a transposon mutant library as attenuated for intracellular replication of *C. burnetii* (31). This gene is not predicted to be involved in NAD biosynthesis itself, but rather encodes a putative short chain dehydrogenase, an enzyme that possesses a predicted NADP binding site that could facilitate NADP(H) regeneration. NADP(H) is a co-factor important in many metabolic functions and plays a key role in resistance to oxidative stress.

Short-chain dehydrogenases/reductases (SDRs) a very large family of enzymes catalysing NADP(H)-dependent oxidation/reduction reactions and consist of around 250 residues (122). This class of enzymes were categorized into a new group of oxidoreducatses in 1970/80s (123,124) and the name SDR was given in 1991 (125). They are found in all life forms from simple organisms to higher multicellular eukaryotes (126), indicating that they are versatile enzymes involved in many metabolic processes. Currently, around 3000 members of the SDR family are well recognized and they act on a wide range of substrates such as steroids, aromatic compounds, sugars, alcohols, and xenobiotics (122). The N-terminal region binds the coenzymes NAD(H) or NADP(H), while the C-terminal region constitutes the substrate binding part of the enzyme (127). Enzymes of this family have two essential structural motifs.

The first conserved domain of SDRs is located on the internal side of α -helical structure and comprises the conserved sequence Ser-Tyr-Lys, which is considered as the active site of the enzyme. The second domain is located around the N terminus and possesses a sequence of Gly-X₃-Gly-X-Gly (glycine rich domain) and is characteristic to all dehydrogenases (128).

The group of SDR superfamily has major differences and possess only low pairwise sequence identity, but they have a common sequence motifs that define the cofactor binding site (TGxxxGxG) and the catalytic tetrad (N-S-Y-K) (127,129). The three-dimensional SDR structures are exactly homologous with a common α/β -folding pattern characterised by a central β -sheet typical of a Rossmann-fold with helices on either side (130). The SDR enzymes are broadly classified into two, namely “classical” and “extended” (122), and they are categorized by their subunit size and sequence patterns at the coenzyme binding site and at a segment N-terminally of the active site region. The classical type consist of around 250 amino acid residues (122) and encompasses oxidoreductases such as steroid dehydrogenases and carbonyl reductases (122) while the extended type has an additional 100-residue domain in the C-terminal region and consists of mainly isomerases such as galactose epimerases, and lyases such as glucose dehydratases. Besides the extended and classical types, the SDR families are further classified as “intermediate”, “complex” and “divergent” based on cofactor and active site sequence motifs (130). All these enzymes use either NAD⁺ or NADP⁺ as a cofactor (131).

2.0. Oxidative stress

2 .1. ROS and RNS

Reactive oxygen species (ROS) are reactive chemical species that contains oxygen molecules such as superoxide, hydroxyl radical and peroxides (132) whereas reactive nitrogen species (RNS) are a group of antimicrobial molecules derived from nitric oxide (NO) (133). They are produced by innate immune cells (phagocytes) that encode two inducible systems for generation of ROS and RNS; the NADP(H) phagocyte oxidase (Phox) and inducible nitric oxide synthase (iNOS) (134). NADP(H) oxidase (nicotinamide adenine dinucleotide phosphate oxidase) is a membrane-bound enzyme complex which is found in the membrane of phagosomes (135). Mononuclear and polymorphonuclear phagocytes express both Phox and iNOS, with higher RNS release from macrophages and higher ROS generation in neutrophils (136). Superoxide radicals (O_2^-) are generated from phagocyte oxidase (Phox) due to transfer of electrons to oxygen. Phox is a multimeric complex which contains both cytosolic

components such as p40^{phox}, p47^{phox} and p67^{phox} and membrane bound components synthesized in phagocytic cells. The active site of Phox is formed by the membrane bound subunits, heterodimeric flavohemeprotein and named as flavocytochrome *b₅₅₈* (136). The cytosolic Phox components are activated during phagocytosis and translocated to the phagosome or cytoplasmic membranes, and assemble with flavocytochrome *b₅₅₈* to form the functional NADP(H) phagocyte oxidase complex (137,138). The transfer of electrons from NADP(H) produce superoxide radicals which spontaneously dismutates to generate hydrogen peroxide (H₂O₂) and form more reactive oxygen intermediates (139,140).

The expression and regulation of iNOS to produce RNS requires the induction by cytokines and microbial products, notably interferon-gamma (IFN- γ) and lipopolysaccharide (LPS) (133). The reaction between the superoxide anion (O₂⁻) and nitric oxide (NO) produces peroxynitrite (ONOO⁻) and initiates RNS regeneration (141). ROS and RNS control infection through their synergistic effects on phagocytosed bacteria. In the early stages of infection Phox is activated to control bacterial replication and at the same time expression of iNOS leading to RNS generation synergistically with ROS to prolong the ROS-mediated effects at the later stage of infection (142,143).

2.2. Mechanisms of cellular damage

Oxidative stress is the elevated level of reactive oxygen species resulting in damage to the molecular processes of an organism. It is an imbalance between the level of ROS and the ability of the system to readily detoxify the reactive intermediates or to repair the resulting damage (134). These imbalances in the normal redox metabolism of the cell can cause deleterious effects on components of the cell, including proteins, lipids, and DNA through the production of peroxides and free radicals. The O₂⁻ and NO radicals catalyse the formation of a variety of toxic intermediates with different reactivity and molecular targets. The ROS mainly target enzymes containing [4Fe-4S]²⁺ including dehydratases of the TCA cycle or DNA. Both O₂⁻ and H₂O₂ can penetrate enzymes and facilitate generation of ferric iron (Fe³⁺) from iron-sulfur centers which causes enzymatic dysfunction and metabolic block (139). Hydrogen peroxide also causes oxidation of free or DNA associated ferrous iron (Fe²⁺), producing hydroxyl radicals and Fe³⁺ via the Fenton reaction (144). The oxidative attack on the DNA backbone due to OH causes base modifications and fragmentation of sugar resulting in mutations or strand breaks respectively. High concentration of hydrogen peroxide causes damage on cellular components whereas low concentration of hydrogen peroxide is deleterious to DNA (134).

Unlike the effect of ROS, the deleterious damage caused by RNS is more complex and is dependent on local redox metabolism (145). Excess NO can react with oxygen and produce nitrosating agents causing DNA damage such as deamination and DNA strand breaks. Peroxynitrite produced by the reaction between superoxide and NO can induce DNA base modifications and strand breaks, oxidation and deamination (146).

2. 3. *C. burnetii* defense mechanisms against oxidative stress

2.3.1. Acid Phosphatase

Many bacterial pathogens have developed a strategy used to evade the host oxidative attack by secreting acid phosphatases which inhibit the production of reactive oxygen intermediates from the host immune cells (147,148). Dai et al. (147) showed that *Francisella tularensis* secretes and translocates acid phosphatase (AcpA) to inhibit the host respiratory burst and ROS response in polymorphonuclear monocytes (PMNs) and allow for survival in a macrophage. *C. burnetii* has evolved a mechanism to suppress the production of ROS in polymorphonuclear monocytes (PMNs) or macrophages through secretion of an acid phosphatase (ACP, CBU0335) (149). Different isolates of *C. burnetii* causing acute infections such as group I isolate Nine Mile as well as those isolates causing chronic disease encode ACP, which is located in the periplasmic space (134). A more recent study by Hill and Samuel (149) showed that *C. burnetii* does secrete ACP, not as previously thought by Siemsen et al. (150). The study also indicated that secretion was essential for an enzyme to subvert the host ROI response by either acting directly on or interfering in the assembly of the NADPH oxidase complex. Several microorganisms used a mechanism to block the formation of Phox assembly using ACPs which causes hydrolyzation of second messengers such as phosphatidylinositol 4,5-bisphosphate (PIP2) or myo-inositol 1,4,5 triphosphate (IP3) required for activation of macrophages (151,152). A previous study showed that *C. burnetii* ACP disrupts tyrosine phosphorylation/dephosphorylation reactions related to the signal transduction pathway culminating in the metabolic burst (153).

2.3.2. Superoxide dismutase (SOD)

Superoxide dismutase (SOD, EC 1.15.1.1) is an enzyme which catalyses the conversion (dismutation) of the superoxide (O_2^-) radical into molecular oxygen (O_2) or hydrogen peroxide (H_2O_2), which is subsequently removed by the enzyme catalase (132). SODs are

classified based on the metal cofactor into three categories; periplasmic Cu/ZnSODs (*sodC*) which binds both copper and zinc, cytoplasmic MnSODs (*sodA*) that binds to manganese and FeSODs (*sodB*) that binds to iron. The cytoplasmic MnSODs (*sodA*) and FeSODs (*sodB*) and periplasmic Cu/ZnSODs (*sodC*) are expressed in *E. coli* as a defence mechanism against both endogenous and exogenous ROS. Cytoplasmic SODs are produced for protection of cellular components against superoxide (134) whereas periplasmic Cu/ZnSODs are synthesized against exogenous superoxide (154).

C. burnetii encodes both a periplasmic Cu/ZnSODs (CBU1822) and cytoplasmic FeSODs (CBU1708) (155,156)). Mutational analysis of cytoplasmic or periplasmic SODs indicated that some pathogens showed no growth defects whereas others are highly sensitive to oxidative stress within the macrophage host. These variations could be due to the differences in repertoires of antioxidant genes and their regulation by these pathogens. The low virulent *C. burnetii* isolate Dugway encodes an inactive Cu/ZnSOD but a functional catalase. This suggests that periplasmic Cu/ZnSOD is more important than catalase in resistance to oxidative stress and that Cu/ZnSOD is essential for protection against exogenous ROS (157). On contrary, in the phylogenetically-related organism *L. pneumophila*, inactivation of the cytoplasmic FeSOD is lethal whereas inactivation of the periplasmic Cu/ZnSOD has a very limited effect on growth (158,159). This is most likely due to the different intracellular compartments occupied by these two pathogens, as the CCV is more similar to a mature lysosome than the vacuole *L. pneumophila* replicates within and *L. pneumophila* is most likely exposed to lower levels of exogenous ROS, so the function of the periplasmic SOD might be dispensable.

2.3.3. Catalase

Catalase is a common enzyme found in nearly all living organisms and protects the cells from oxidative damage caused by ROS through the decomposition of hydrogen peroxide to water and oxygen (160). It consists of four iron-containing heme groups which allows the enzyme to react with the hydrogen peroxide (161). Catalases are classified as monofunctional catalases, bifunctional catalases and manganese-containing catalases (160). Many organisms such as *Vibrio cholerae* express more than one type of catalase regulated by OxyR (162). The *V. cholerae oxyR* deletion mutant was not only sensitive to H₂O₂, but also showed a growth defect in culture suggesting that catalases are critical for antioxidant defense of *V. cholerae* (162). This study also indicated that both *katG* and *katB* genes in *V. cholerae* play a vital role in detoxifying

H₂O₂ and resistance to oxidative stress. *L. pneumophila* expresses both a periplasmic KatA and cytosolic KatB catalases. They are differentially induced, being expressed in either stationary (katA) phase or during exponential growth (katB) (163,164). Inactivation of both *katA* and *katB* in *L. pneumophila* resulted in greater susceptibility to exogenous hydrogen peroxide and reduced virulence in macrophage-like cells (163), suggesting that both catalases are required for the biphasic life cycle and aerobic growth of *L. pneumophila*. The majority of *C. burnetii* isolates encode a non functional catalase, except a few isolates that express a single functional catalase, KatE (CBU0281) (35). Both the low-virulent isolate Dugway of group VI and the group IV isolate K (Q154) isolated from the heart valve of endocarditis patient, are the two *C. burnetii* isolates encoding a functional KatE (165,166). However, *C. burnetii* isolates Nine Mile and Henzerling (group I and II) as well as isolate G (group V) express a non-functional KatE and result in acute and chronic infections respectively. Functional expression of catalase in *C. burnetii* leads to improved protection against oxidative stress both *in vitro* and *in vivo*, but over expression causes a negative growth phenotype (134), suggesting that the role of catalase in *C. burnetii* is complex.

2.3.4. Repair of oxidized cysteine residues

In addition to encoding enzymes that convert ROS and RNS to less toxic intermediates, most organisms have evolved repair mechanisms for oxidatively damaged proteins (134,167). Given they contain electron-rich sulfur atom in their side chain, sulfur-containing amino acids such as cysteine and methionine are particularly sensitive to oxidative damage by ROS and RNS. The oxidized cysteine residues are reduced to the thiol state by various oxidoreductases, including thioredoxins (Trxs) and glutaredoxins (Grxs) (167), whereas the repair of oxidized methionine is carried out by methionine sulfoxide reductases (Msrs) (168). Thioredoxins (Trxs) are ubiquitous small soluble redox proteins known to be present in all organisms and act as antioxidants by catalysing thiol-disulfide redox reactions (169). They are kept in the reduced state by the help of the enzyme thioredoxin reductase (TrxB), a flavoenzyme which specifically facilitates the NADP(H)-dependent reduction of thioredoxin (170). Trxs contain two conserved cysteines (CXXC) on their active site and the amino-terminal of this motif is mostly present as a thiolate under normal conditions. The reaction begins with the attack of the first motif onto the oxidized group of the substrate (171). The second cysteine residue forms a complex disulfide bond with the first cysteine thereby transferring 2 electrons to the substrate which is now in its reduced form. Oxidized Trx is then reduced by thioredoxin reductase, a

nicotinamide adenine dinucleotide phosphate (NADP(H))-dependent flavoenzyme (171). Thioredoxin reductase uses NADP(H) to maintain levels of reduced thioredoxin through the similar mechanism used by glutathione reductase to maintain GSH levels (167). Genome annotations indicate that *C. burnetii* encodes a thioredoxin reductase (CBU2087) (62). *E. coli* expresses two Trxs, Trx1 and Trx2. Trx1 is required for reduction of disulfide bonds that form in the catalytic intermediates of various enzymes such as phosphoadenosine-phosphosulfate (PAPS) reductase, ribonucleotide reductase, thiol peroxidase and bacterioferritin comigratory protein (Bcp). Many proteins involved in several biological processes such as transcription and regulation of cell shape, biosynthesis and energy metabolism need Trx1 for their proper function. These proteins have cysteine residues which makes them more susceptible to oxidation and they are dependent on Trx1 to repair oxidative damage (172).

Glutaredoxins (Grxs) are small oxidoreductases, structurally related to thioredoxins, particularly in their active site domain, and these enzymes reduce disulfides in target protein and are regenerated by glutathione (GSH; a low-molecular-weight thiol) (173,174). The majority of Grxs possess a CXXC active motif and are known as dithiol enzymes whereas those containing only one cysteine (CXXS) in their catalytic motif are referred to as monothiol enzymes (174). Like thioredoxin, which functions in a similar way, glutaredoxin reacts with oxidized proteins when the disulfided protein substrate is reduced. Dithiol Grxs are reduced by glutathione (GSH) and reacts with the first cysteine residue in the CXXC motif to form a mixed-disulfide intermediate. The complex disulfided intermediate reacts with the second GSH intermediate resulting in a formation of a reduced Grx and an oxidized glutathione molecule (GSSG). Oxidized glutathione is reduced to glutathione by NADP(H)-dependent glutathione reductase (167). Bioinformatics analysis and genome annotations suggest that *C. burnetii* encodes genes to synthesize glutathione (glutathione genes) and the corresponding glutaredoxin (grxC , CBU1520) (134).

2.3.5. Repair of oxidized methionine residues

Amino acids containing sulfur residues such as methionine are also sensitive to oxidation by reactive oxygen species and the repair of oxidized methionine is carried out by methionine sulfoxide reductases (Msrs) (168). The sulfur atom located in the thioether side chain of methionine is oxidized to sulfoxide which converts methionine into methionine sulfoxide (Met-O) (175). Met-O exists in two diastereoisomeric forms, methionine-(R)-sulfoxide (Met-R-O) and methionine-(S)-sulfoxide (Met-S-O) (176) that can be further oxidized into

methionine sulfone (Met-O₂) (175). There are three types of Msrs, methionine sulfoxide reductase A (MsrA) which reduce both free Met-S-O and Met-S-O in proteins (177), MsrB that primarily acts on protein-bound Met-R-O (178) and MsrC that can only reduce free Met-R-O (179,180).

Both MsrA and MsrB have similar catalytic mechanisms for reduction of methionine sulfoxide, but they differ in their structure and sequence (181-184). The reduction reaction starts with the attack of Met-O substrate by Cys residue (CysA) resulting in sulfenic acid (–SOH) group on CysA and generation of reduced methionine. Secondly, a nucleophilic Cys residue (CysB) reacts with CysA–SOH and forms an intramolecular disulfide intermediate and water. Finally, this intramolecular disulfide intermediate is reduced by the help of a Trx protein and leads to regeneration of active Msr enzyme (167). To date MsrA is the best studied Msrs, and is required for resistance to oxidative stress and virulence by many pathogenic organisms such as *Leishmania major* (185), *Trypanosoma spp* (186) *Mycoplasma genitalium* (187,188), *Enterococcus faecalis* (189) and *Salmonella typhimurium* (190). In addition, a study by Singh et al. (191) also showed that MsrA is crucial for resistance against oxidative stress in *Staphylococcus aureus*. *C. burnetii* encodes a methionine sulfoxide reductase, MsrA (CBU1306) which acts on both free Met-S-O and protein bound Met-S-O (134), but its importance in pathogenesis has not been investigated.

2.3.6. Peroxiredoxins

Peroxiredoxins are a ubiquitous family of antioxidant enzymes which degrade hydrogen peroxide (H₂O₂), organic hydroperoxides (ROOH) and peroxynitrite into H₂O and their corresponding alcohols (134). They are usually referred to as alkyl hydroperoxide reductase (AhpC) in pathogenic bacteria (192). AhpCs encode two conserved cysteine residues facilitating degradation of hydroperoxides in two basic procedures; the reaction is initiated by oxidation of redox-active cysteine (the peroxidatic cysteine) in the active site to sulfenic acid by the peroxide substrate and forms a disulfide bound with the second conserved, resolving cysteine residue (193). The regeneration of the sulfenic acid back to a thiol state is carried out by thiols such as thioredoxins or possibly glutathione and ascorbic acid (194). A number of bacteria such as *E. coli* or *S. typhimurium* encode flavoproteins together with peroxiredoxin which serve as disulfide reductases for recycling of the conserved cysteine residues in the peroxiredoxin such as AhpC/AhpF system (192). Similar to *L. pneumophila*, *C. burnetii* expresses AhpC1 (CBU1706) and AhpC2D (CBU1477/1478) which are alkyl hydroperoxide

systems involved in peroxide scavenging (134). *C. burnetii* AhpC1 and AhpC2D are closely related with two alkyl hydroperoxidases AhpC/AhpD of *M. tuberculosis* (195) and NADP(H)-dependent TrxR/Trx activated AhpC of *Helicobacter pylori* (196).

The high homology of AhpC systems between *L. pneumophila* and *C. burnetii* suggests that these two pathogens may share similar defence mechanism against oxidative damage caused by ROS (197). Mutations of either *ahpC1* or *ahpC2* of *L. pneumophila* resulted in greater susceptibility to exogenous peroxides, but didn't show any growth defects in both normal culture conditions and *in vivo* (197,198). The *C. burnetii* *ahpC2D* operon has a similar genome organisation to the one found in *B. abortus*, *B. melintensis*, *M. bovis* and *M. leprae* (134). In *M. tuberculosis*, AhpC undergoes an unusual regulatory cycle and is expressed by static bacilli only (199). Expression of antioxidant genes are associated with dormancy in *M. tuberculosis* or latency in other pathogens. Nevertheless, *ahpC2* of *C. burnetii* is supposed to be expressed during oxidative stress, but its contribution in protection against oxidative stress inside the CCV or during various stages of replication stages is unknown (200).

2.4. Thesis aims

We hypothesize that *C. burnetii* intracellular replication and survival requires NAD synthesis and NADP(H)-regeneration. The previously conducted transposon mutagenesis screen (31) identified that roughly 10% of the transposon mutants exhibited either nil or reduced intracellular replication within human HeLa cells. Two genes identified in this previous study, *nadB* and *cbu_1276*, encoded proteins with predicted metabolic functions related to either NAD synthesis or regeneration of NAD-containing compounds (31). Three independent mutants were identified for *nadB*, all of which showed reduced intracellular growth at six days post-infection, suggesting an important role for NadB in intracellular replication. The *sdrA* transposon mutant also showed reduced replication at six days post infection inside Hela cells. This gene encodes a putative short chain dehydrogenase, an enzyme containing a predicted NADP binding site that could facilitate NADP(H) regeneration by *C. burnetii*. Given the apparent absence of the oxidative branch of the pentose phosphate pathway in *C. burnetii* (201), and the lack of knowledge of how the bacterium therefore regenerates sufficient NADP(H), this gene was also particularly interesting.

Therefore, the overall aim of this study is to investigate the role of these metabolic genes in the pathogenesis of *C. burnetii*. Specifically, this thesis aims to:

- confirm the requirement for *nadB* and *sdrA* for *C. burnetii* replication in host cells

- functionally characterise NadB and SdrA, using sophisticated biochemical and genetic techniques
- identify the molecular mechanism whereby NadB and SdrA contribute to *C. burnetii* survival and replication inside host cells
- investigate the feasibility of inhibiting one or both of these enzymes during *C. burnetii* intracellular replication

Chapter 2. *De novo* NAD synthesis is required for intracellular replication of *Coxiella burnetii*, the causative agent of the neglected zoonotic disease Q fever

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De novo NAD synthesis is required for intracellular replication of *Coxiella burnetii*, the causative agent of the neglected zoonotic disease Q fever

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Coxiella burnetii is an intracellular Gram-negative bacterium responsible for the important zoonotic disease Q fever. Improved genetic tools and the ability to grow this bacterium in host cell-free media has advanced the study of *C. burnetii* pathogenesis, but the mechanisms that allow it to survive inside the hostile phagolysosome remain incompletely understood. Previous screening of a transposon mutant library for replication within HeLa cells has suggested that *nadB*, encoding a putative L-aspartate oxidase required for *de novo* NAD synthesis, is needed for intracellular replication. Here, using genetic complementation of two independent *nadB* mutants and intracellular replication assays, we confirmed this finding. Untargeted metabolite analyses demonstrated key changes in metabolites in the NAD biosynthetic pathway in the *nadB* mutant compared with the WT, confirming the involvement of NadB in *de novo* NAD synthesis. Bioinformatic analysis revealed the presence of a functionally conserved arginine residue at position 275. Using site-directed mutagenesis to substitute this residue with leucine, which abolishes the activity of *Escherichia coli* NadB, and expression of WT and R275L GST-NadB fusion proteins in *E. coli* JM109, we found that purified recombinant WT GST-NadB has L-aspartate oxidase activity and that the R275L NadB variant is inactive. Complementation of the *C. burnetii nadB* mutant with a plasmid expressing this inactive R275L NadB failed to restore replication to WT levels, confirming the link between *de novo* NAD synthesis and intracellular replication of *C. burnetii*. This suggests that targeting this prokaryotic-specific pathway could advance the development of therapeutics to combat *C. burnetii* infections.

Coxiella burnetii is a Gram-negative intracellular bacterium that causes the zoonotic disease Q fever (1), which primarily spreads to humans from livestock such as cattle and sheep. In humans, infection can present as an acute disease with systemic

signs including pneumonia and hepatitis. A chronic infection, characterized by endocarditis and chronic fatigue syndrome, may subsequently develop (1, 2). In 2007–2010 an outbreak in the Netherlands resulted in more than 4000 human cases, the culling of tens of thousands of animals, and cost in excess of 300 million Euros to control (3). *C. burnetii* primarily targets macrophages and enters via conventional phagocytosis (4). Once trafficked through the endocytic pathway, the bacterium switches from the small-cell variant form to the actively replicating large-cell variant form (5, 6) and activates the maturation of a phagolysosome-like compartment known as the *Coxiella*-containing vacuole (CCV),² where it replicates freely (7–9).

The relatively recent development of axenic growth medium for *C. burnetii* (10) and improved techniques for genetic manipulation (11) facilitated identification of the Dot/Icm type 4 secretion system (T4SS) (responsible for translocating at least 130 effector proteins) as essential for replication inside cells (12–14). However, much less is known about the Dot/Icm type 4 secretion system-independent factors that are required for replication inside cells. Recent screening of a transposon mutant library (12) for replication within human HeLa cells demonstrated that ~10% of transposon mutants were attenuated for growth, including three independent mutants with transposon insertions into *nadB*. The *nadB* gene encodes a putative L-aspartate oxidase that is essential for the canonical prokaryotic *de novo* NAD biosynthetic pathway, in which NadB converts aspartate to iminoaspartate, which is then converted to quinolinate and eventually NAD via downstream enzymes (15). NAD is an important cofactor for many core metabolic reactions (16, 17). Intracellular NAD⁺/NADH ratio is key to the maintenance of an adequate metabolic status and cell survival (18) and may be especially important for *C. burnetii*, because the phagolysosome is a highly oxidative environment.

In this study we genetically complemented two *nadB* mutants and performed quantitative and qualitative intracellular replication assays to confirm that *nadB* is required for intracellular replication of *C. burnetii*. We then used a combination

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This article contains Figs. S1–S4.

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² The abbreviations used are: CCV, *Coxiella*-containing vacuole; RT, retention time; NaMN, nicotinic acid mononucleotide; GST, glutathione S-transferase; OAA, oxaloacetate; TCA, tricarboxylic acid; NMII, Nine Mile phase II; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; qPCR, quantitative PCR; DAPI, 4',6'-diamino-2-phenylindole.

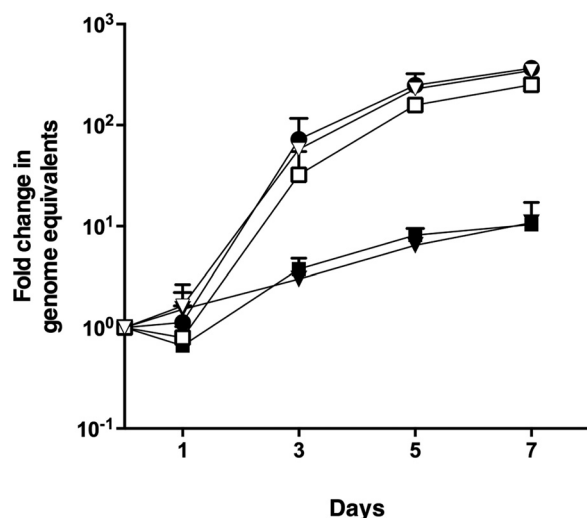


Figure 1. *nadB* is essential for efficient intracellular replication of *C. burnetii*. Intracellular replication of *C. burnetii* NMII WT (closed circles), *nadB*::Tn 7-G9 (closed squares), *nadB*::Tn 7-G9 complemented strain (open squares), *nadB*::Tn 23-H5 (closed inverted triangles), and *nadB*::Tn 23-H5 complemented strain (open inverted triangles) was measured over 7 days. The fold increases in GE relative to the inoculum were determined by *ompA*-specific qPCR and are represented here as the means $\pm$ S.D. of three independent infections at days 1, 3, 5, and 7 postinfection. Both *nadB*::Tn mutants were significantly reduced in growth compared with *C. burnetii* NMII at days 3, 5, and 7 ($p < 0.05$, paired *t* test).

of sophisticated biochemical and genetic techniques, including GC/MS and LC/MS, recombinant protein purification, enzymatic assays, and site-directed mutagenesis, to demonstrate that *de novo* NAD biosynthesis is required for *C. burnetii* replication inside human cells.

Results

nadB is required for efficient intracellular replication of *C. burnetii* and normal CCV biogenesis

Following the identification in our initial screen of *nadB* as a candidate gene required for intracellular replication, we aimed first to confirm this role. Two independent transposon mutants, *nadB* 7-G9::Tn and *nadB* 23-H5::Tn, with the transposon inserted 900 and 1080 bp into the ORE, respectively, were clonally isolated; the presence of the transposon was confirmed by PCR; and each mutant was complemented with plasmid expressing full-length 3xFLAG::NadB, with the expression of protein of the predicted molecular weight confirmed using anti-FLAG immunoblotting (Fig. S1). Intracellular growth assays in human HeLa CCL2 cells were performed, and both *nadB* mutants had significantly reduced growth, with only a 10-fold increase in genome equivalents at 7 days postinfection, in contrast to WT *C. burnetii* that increased by over 100-fold in the same period (Fig. 1). Complementation with the pJB-Kan:3xFLAG vector expressing 3xFLAG-NadB restored growth to WT levels (Fig. 1), demonstrating conclusively that *nadB* is required for efficient *C. burnetii* replication. Qualitative assays using immunofluorescence microscopy examined the effect of transposon insertion into *nadB* on the size and number of the CCVs. Both *nadB* mutants displayed much smaller CCVs (stained green) containing very few bacteria

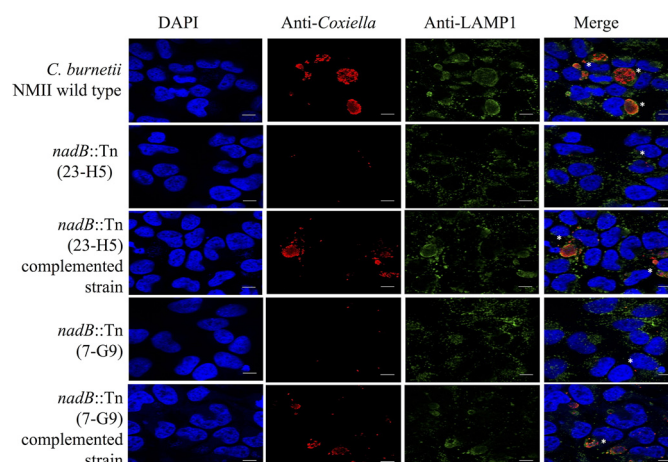


Figure 2. *nadB* is required for normal CCV formation. Shown are representative micrographs (three independent experiments) of HeLa cells infected for 3 days with *C. burnetii* NMII, the *nadB*::Tn 23-H5 mutant, the *nadB*::Tn (23-H5) complemented strain, the *nadB*::Tn 7-G9 mutant, and the *nadB*::Tn (7-G9) complemented strain. The cells were stained with anti-*Coxiella* antibody (red), anti-LAMP1 antibody (green), and DAPI (blue), and white asterisks indicate representative CCVs. Note that the CCVs containing the *nadB* mutant appear smaller and contain few bacteria compared with either the WT NMII WT strain or the complemented strains. The scale bars represent 10 μ m.

(stained red), whereas plasmid complementation of the *nadB* mutants restored the WT phenotype (Fig. 2). Because similar results were obtained for both *nadB* mutants, *nadB* 23-H5::Tn and the corresponding complemented strain were used for the remainder of experiments and are referred to as the *nadB* mutant and the complemented *nadB* mutant.

nadB is required for normal NAD biosynthesis

To examine the effect of the loss of *nadB* in *C. burnetii* cultured in axenic medium, we performed GC/MS analysis to compare the abundance of metabolites within the *de novo* NAD biosynthetic pathway (Fig. 3A) between the *nadB* mutant, the complemented *nadB* mutant, and WT *C. burnetii* grown in axenic medium. Axenic cultures of *C. burnetii* were rapidly quenched, and polar metabolites were extracted, derivatized, and analyzed using GC/MS. Metabolites were identified from representative chromatograms by retention time (RT) and fragmentation pattern, and log transformation and median normalization were performed before the abundance of each metabolite was compared. As predicted, the loss of NadB resulted in a significantly increased abundance of aspartate in the *nadB* mutant, with a 4.1 log₂ fold increase compared with WT and a 4.02 log₂ fold increase compared with the complemented mutant (Fig. 3B). Furthermore, downstream metabolites were significantly decreased in the *nadB* mutant, with quinolate decreased by 2.04 log₂ fold and 1.6 log₂ fold in the mutant compared with WT and the complemented mutant, respectively (Fig. 3B). Similarly, nicotinic acid mononucleotide (NaMN) was decreased by 1.24 and 2.35 log₂ fold in the *nadB* mutant, as compared with WT and the complemented mutant, respectively (Fig. 3B).

To examine whether the loss of *nadB* affected the abundance of the metabolites downstream of the predicted entry of nicotinate via a salvage pathway, namely NAD, NADP, and NADH (Fig. 3A), LC/MS analysis was performed. Metabolites were

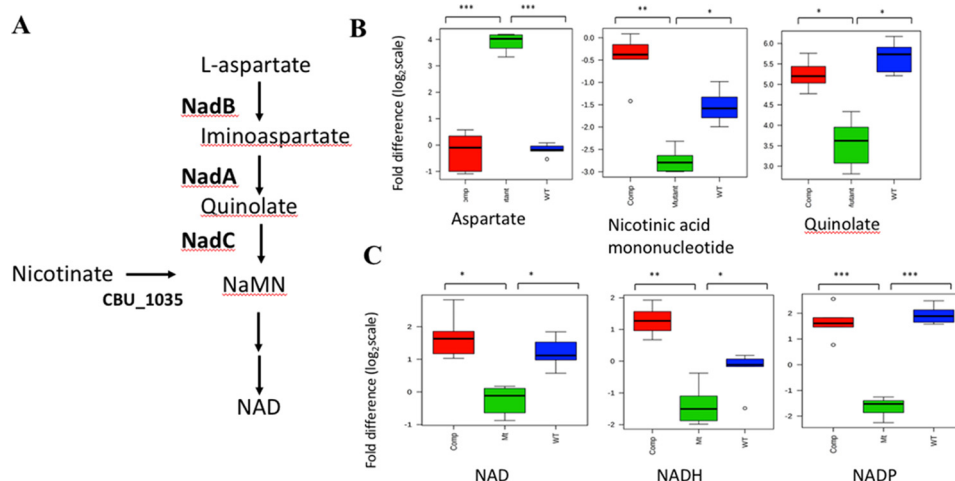


Figure 3. A, the *C. burnetii* NAD biosynthetic pathway. B, disruption of *nadB* alters the level of *de novo* NAD biosynthetic pathway metabolites. Red, complemented *nadB* mutant; green, *nadB* mutant; blue, WT *C. burnetii* NMII. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (BH-adjusted *t* test). Fold difference is presented as log₂ scale. C, disruption of *nadB* reduces the abundance of NAD-containing metabolites. NAD, NADH, and NADP were all significantly reduced in the *nadB* mutant compared with WT *C. burnetii* and the complemented *nadB* mutant. Metabolites were identified using LC/MS, data were median-normalized, and metabolites were identified by reference to authentic standards. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (BH-adjusted *t* test). Fold difference is presented as log₂ scale.

extracted from rapidly quenched axenically cultured *C. burnetii* and polar metabolites were analyzed by LC/MS, before median normalization and identification of metabolites using authentic standards. The relative abundance of each metabolite was compared. The *nadB* mutant displayed 1.32 and 2.07 log₂ fold decreases in NAD levels relative to the WT and the complemented mutant strains, respectively (Fig. 3C). The NADH abundance in the *nadB* mutant was decreased by 1.06 and 2.58 log₂ fold compared with the WT and the complemented strain, respectively (Fig. 3C). Similarly, NADP was decreased in the *nadB* mutant by 3.60 and 3.37 log₂ fold compared with the WT and the complemented strain, respectively (Fig. 3C). Overall, our metabolite analysis demonstrated an effect on *de novo* NAD synthesis only in the absence of *nadB*, demonstrating that NadB is required for NAD biosynthesis and that salvage pathways are not sufficient, even in rich axenic medium, to restore the levels of NAD-containing metabolites in the *nadB* mutant to those present in WT *C. burnetii*.

Mutation of the critical amino acid residue Arg²⁷⁵ of NadB abolishes enzymatic function in vitro

To investigate whether the enzymatic function of NadB, and therefore NAD biosynthesis, was crucial to the ability of *C. burnetii* to replicate inside cells, we first investigated the function of NadB *in vitro*. Recombinant glutathione *S*-transferase (GST)-NadB (GST-NadB) was expressed in and purified from *Escherichia coli* JM109, with GST alone purified as a negative control. To confirm that NadB had the expected L-aspartate oxidase activity, a two-step *in vitro* enzyme assay was carried out. *In vitro*, in the absence of the downstream enzyme NadA, the iminoaspartate produced from L-aspartate by NadB catalysis spontaneously hydrolyzes to oxaloacetate (OAA) (19, 20). This OAA can then be detected by performing a second step, in which commercial malate dehydrogenase is used to reduce OAA to malate, and the change in absorbance at 340 nm (resulting from concurrent NADH oxidation during OAA reduction) is measured. The absorbance will only decrease in

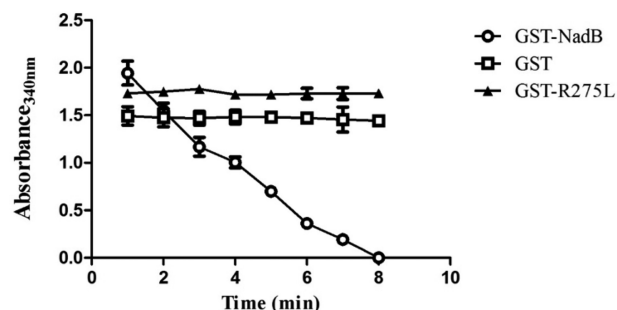


Figure 4. Recombinant GST-NadB possesses L-aspartate oxidase activity that is abolished by mutation of Arg²⁷⁵ to Leu. The graph depicts the second step of the two-step assay, demonstrating the change in absorbance at 340 nm resulting from oxidation of NADH and reduction of OAA in the presence of commercial malate dehydrogenase, if the first step of the assay produced iminoaspartate (which spontaneously hydrolyzes to OAA). A decrease in absorbance will only occur if the protein used in the first step possesses L-aspartate oxidase activity. Proteins used in the first step were GST-NadB (circles), GST (squares), and GST-R275L-NadB (triangles). The graph represents the mean $\pm$ S.D. of three independent experiments.

the second step of the assay if iminoaspartate is produced by the enzyme used in the first step of the assay.

When GST-NadB was used in the first step of the assay, a fall in absorbance at 340 nm to zero was observed in the second step (Fig. 4), indicating that NadB had L-aspartate oxidase activity (because it produced iminoaspartate in the first step). In contrast, when GST alone was used to catalyze the first step of the assay, no change in absorbance over time was observed in the second step of the assay.

To link the enzymatic function of NadB with its role in intracellular replication and rule out a secondary, nonenzymatic role in intracellular replication, we aligned the amino acid sequence of *C. burnetii* NadB with that of *E. coli* NadB (Fig. 5) and identified a conserved arginine in position 275 (position 290 in *E. coli*). In *E. coli* NadB, mutation of this arginine to leucine abolishes the enzymatic activity (21). We performed site-directed mutagenesis, then expressed and purified recombinant *C. burnetii* GST-NadB-R275L (GST-R275L), and performed the two-step enzyme assay. When GST-R275L was used to cat-

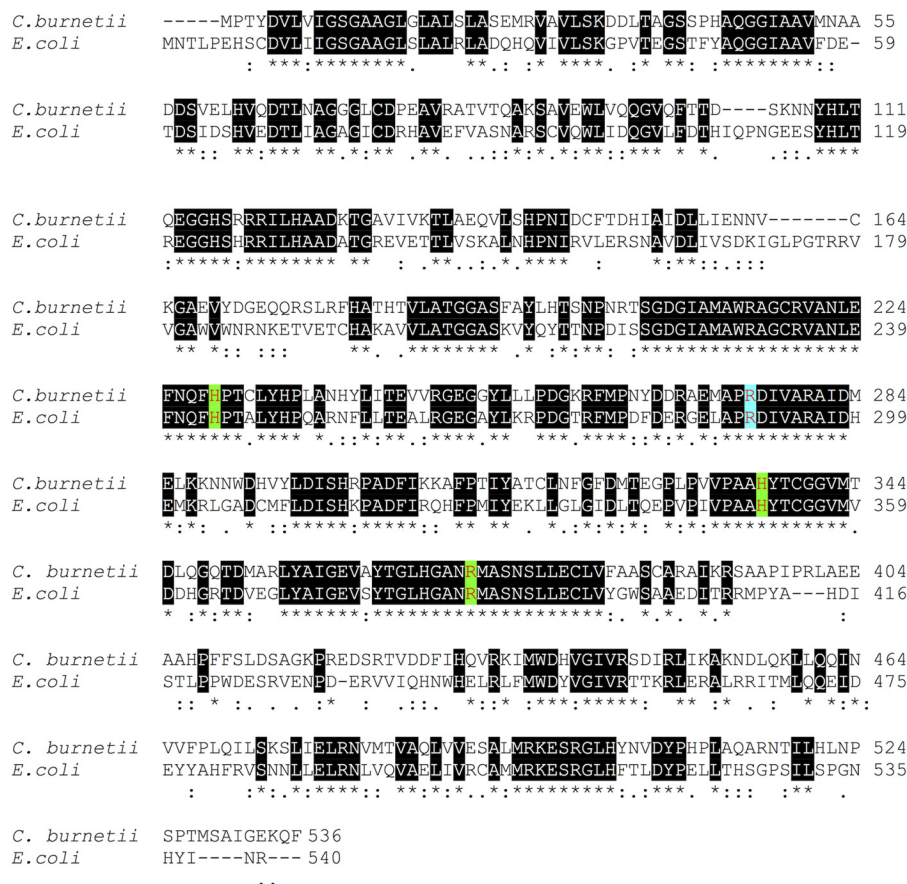


Figure 5. Key amino acid residues are conserved between *E. coli* NadB and *Coxiella* NadB. Protein sequence alignment was performed using Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>; Ref. 33). (Please note that the JBC is not responsible for the long-term archiving and maintenance of this site or any other third party hosted site.) The conserved amino acids were predicted to be involved substrate binding (His²⁴⁴, His³³⁶, and Arg³⁷¹, highlighted green), and the catalytic site (Arg²⁷⁵, highlighted blue) was identified in *Coxiella* NadB. Numbers on the right side indicate position of amino acids for both organisms. Conserved regions are indicated by black shading and asterisks; regions of similarity are indicated by colons.

analyze the first step of the reaction, no change in absorbance was observed for the second step (Fig. 4), indicating that iminoaspartate was no longer produced in the first step of the assay, and thus that the mutant R275L protein had lost its L-aspartate oxidase function.

***De novo* NAD biosynthesis is required for intracellular replication of *C. burnetii* in both epithelial and macrophage-like cells**

To confirm whether *de novo* NAD synthesis is required for intracellular replication of *C. burnetii* and to investigate whether the type of host cell affects this requirement, we complemented the *nadB* mutant with the pJB-Kan:3xFLAG plasmid expressing 3xFLAG-R275L-NadB and performed quantitative and qualitative replication assays using both HeLa cells and human macrophage-like THP-1 cells. In HeLa cells, complementation of the *nadB* mutant with this inactive R275L-NadB resulted in a similar phenotype to that of the original *nadB* mutant in HeLa cells, with a fold increase in genome equivalents of only 11.7 after 7 days (Fig. 6A). In contrast, as in our original experiments, complementation with the pJB-Kan:3xFLAG plasmid expressing WT 3xFLAG-NadB rescued the intracellular growth defect (Fig. 6A). Similarly, in the macrophage-like THP-1 cells, both WT and the complemented *nadB* mutant were more than 100-fold greater in number at day 7

than either the *nadB* mutant or the complemented strain expressing the inactive R275L-NadB (Fig. 6B). Expression of both NadB and R275L-NadB by the complemented strains during THP-1 infection was confirmed by immunoblotting (Fig. S2). Immunofluorescent microscopy revealed a similar CCV phenotype for the R275L complemented strain to that of the original *nadB* mutant, with very small CCVs containing few bacteria, in both HeLa cells and THP-1 cells (Fig. 7). Crucially, these results demonstrate that active NadB, and therefore a functional *de novo* NAD biosynthesis pathway, are required for intracellular replication of *C. burnetii* in both professional phagocytes and epithelial cells.

The overall metabolic profile of the *nadB* mutant differs from that of WT and the complemented mutant

NAD and its derivatives are important cofactors in many metabolic reactions within cells. To investigate why the loss of *de novo* NAD synthesis may affect intracellular replication, we interrogated our GC/MS and LC/MS data to analyze the effect of the loss of NadB on the overall metabolite profile of the *nadB* mutant, as compared with the metabolite profiles of the WT and the complemented *nadB* mutant. In total, we identified 88 metabolites using LC/MS and 110 metabolites using GC/MS and performed pairwise comparisons of the abundance of each metabolite between WT and the *nadB*

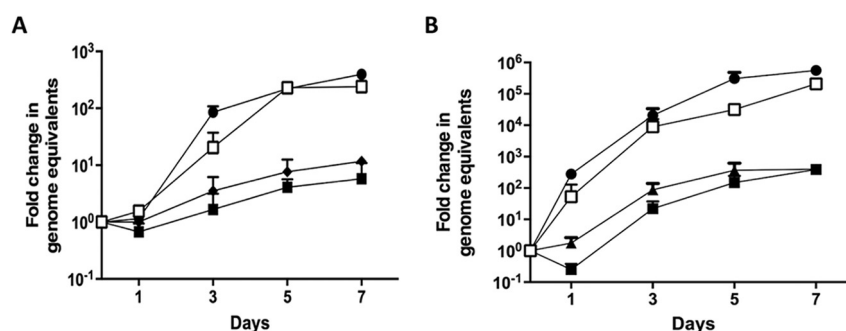


Figure 6. Complementation of the *nadB*::Tn mutant with a plasmid expressing the inactive R275L-NadB did not rescue the intracellular growth defect in either HeLa cells (A) or THP-1 cells (B). Intracellular replication of *C. burnetii* was measured over 7 days. The fold increases in GEs relative to the inoculum were determined by *ompA*-specific qPCR and are represented here as the means $\pm$ S.D. of three independent infections at days 1, 3, 5, and 7 postinfection. Both the *nadB* mutant (closed squares) and the R275L complemented *nadB* mutant (closed triangles) were significantly reduced in growth in HeLa cells compared with *C. burnetii* NMII WT (closed circles) at days 5 and 7 ($p < 0.05$, paired *t* test) and at all time points in THP-1 cells ($p < 0.05$, paired *t* test). Open squares represent the original complemented *nadB* mutant.

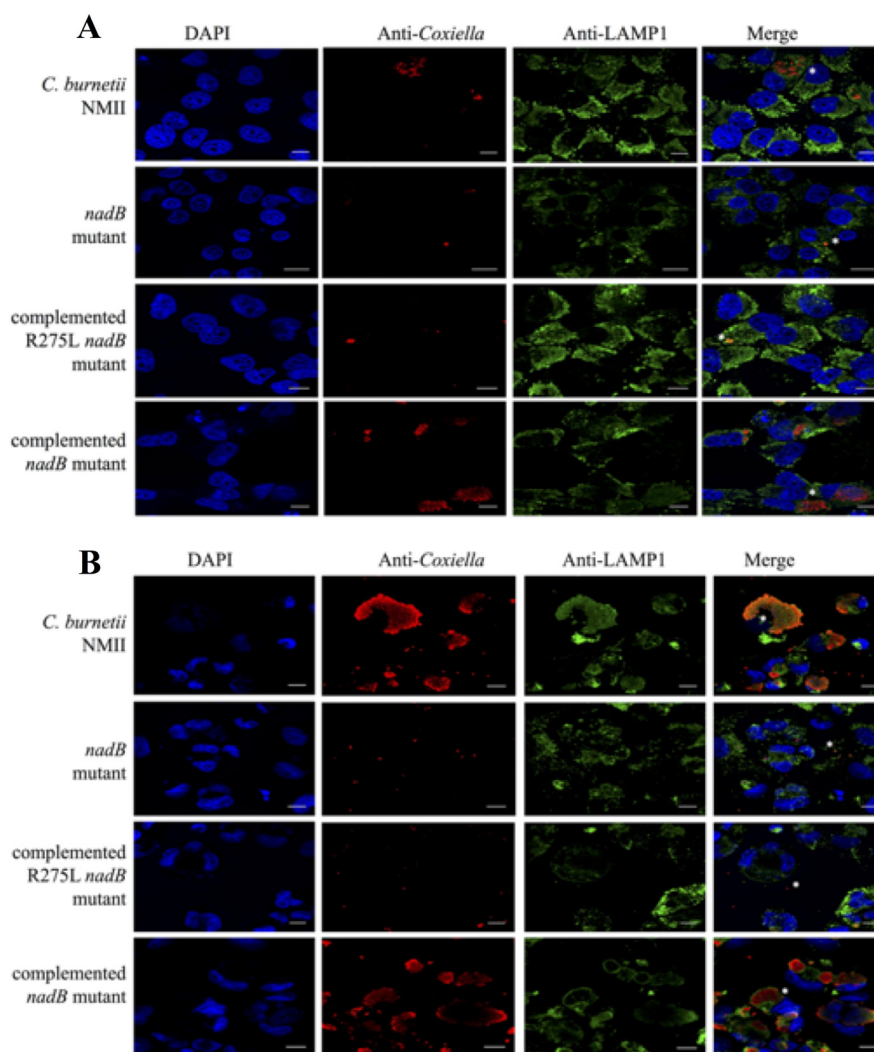


Figure 7. Enzymatically active NadB is required for normal CCV formation and *C. burnetii* growth inside cells. Representative micrographs (three independent experiments) of HeLa cells (A) and THP-1 cells (B) infected with *C. burnetii* strains for 3 days are shown. The cells were fixed and stained with anti-*Coxiella* (red), anti-LAMP1 (green), and DAPI (blue), and white asterisks indicate representative CCVs. Note that WT *C. burnetii* NMII and the complemented *nadB* mutant produced large CCVs containing numerous bacteria, whereas both the *nadB* mutant and the R275L complemented *nadB* mutant displayed smaller CCVs containing very few bacteria. The scale bars represent 10 μ m.

mutant and between the *nadB* mutant and the complemented strain. Metabolites were identified using standard retention time and mass spectra and by comparison with authentic standards.

Principal component analysis demonstrated good separation between the *nadB* mutant and the other two strains, with (as expected) overlap between the complemented mutant and WT (Fig. S3), demonstrating that the loss of *nadB* affected the over-

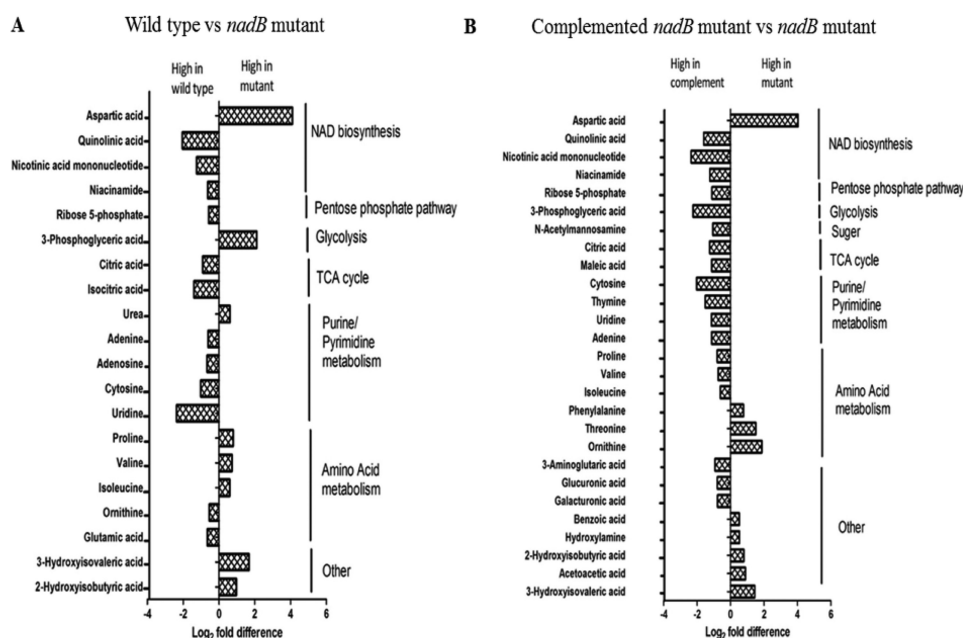


Figure 8. Metabolites differing significantly ($p < 0.05$, BH-adjusted t test) in abundance between WT and the *nadB* mutant (A) and between the complemented *nadB* mutant and the *nadB* mutant (B) in the GC/MS analysis. Metabolites from all strains were identified from representative chromatograms using RTs. The data were log-transformed and median-normalized before analysis. The data represent the average $\log_2$ fold change from six biological repeats.

all metabolic profile. Pairwise comparison of metabolites from the GC/MS analysis identified statistically significant differences in the abundances of 20 metabolites between the *nadB* mutant and WT, and 27 metabolites differed between the *nadB* mutant and the complemented strain. (Fig. 8). Fold differences were similar, with the differences for the WT and mutant ranging from 4.1 to $-2.36 \log_2$ fold, and the differences for the complemented strain *versus* the mutant the fold differences varied by 4.02 to $-2.35 \log_2$ fold. In addition to the previously discussed differences in metabolites directly related to NAD biosynthesis, significant differences were seen in metabolites within glycolysis, the pentose phosphate pathway, the tricarboxylic acid (TCA) pathway, DNA synthesis, and amino acid metabolism.

The TCA cycle intermediate citrate was elevated in abundance in both WT and the complemented mutant strain compared with the *nadB* mutant, with a relative elevation in isocitrate abundance in WT as well. The pentose phosphate pathway intermediate ribose 5-phosphate was relatively elevated in both WT and the complemented mutant strain. The amino acid abundances varied between the two comparisons, with differing patterns observed between WT and the complemented *nadB* mutant. Significant elevations in a number of nucleobases, nucleosides, and nucleotides were observed in WT and/or the complemented strain compared with the *nadB* mutant. Overall, disrupting NAD synthesis had significant effects on the levels of a number of metabolites, including those found in central carbon metabolic pathways.

Discussion

NAD and its phosphorylated and reduced derivatives such as NADP, NADPH, and NADH, are crucial cofactors in many core cellular metabolic reactions (16), including glycolysis and the

TCA cycle. Broadly speaking, prokaryotes synthesize NAD via a three-step pathway from L-aspartate (Fig. 3A), although there are exceptions, such as *Haemophilus influenzae*, that lacks *de novo* synthesis and instead relies on salvage pathways. Most eukaryotes, including humans, synthesize NAD using a five-step pathway from tryptophan (16), suggesting that the bacterial NAD biosynthetic pathway could be a suitable therapeutic target. We have applied a combination of genetic and biochemical techniques to demonstrate the key role of this pathway in *C. burnetii* pathogenesis, supporting the identification of this pathway as a novel drug target.

A number of NAD salvage pathways exist in prokaryotes, with the most common converting nicotinamide to nicotinic acid (*E. coli* enzyme PncA) and then to NaMN (*E. coli* enzyme PncB) (16). Downstream of NaMN the *de novo* biosynthetic and salvage pathways converge, and NaMN is converted to nicotinic acid adenine dinucleotide (bacterial enzyme NadD) and then to NAD (bacterial enzyme NadE) (22). *C. burnetii* lacks the Pnc salvage pathway and instead appears to only possess *cbu_1035*, which encodes a putative nicotinate phosphoribosyltransferase (23) that is predicted to convert scavenged nicotinic acid to NaMN (Fig. 3A). In our study, we observed that the loss of NAD *de novo* synthesis can be overcome *in vitro* (Fig. S4), demonstrating that this salvage pathway must be active at least *in vitro* and that *C. burnetii* can scavenge nicotinic acid from the rich axenic medium used to culture the cells. Recent analysis of lysosomal metabolites (24) detected nicotinic acid within this cellular compartment, suggesting that it may be present within the CCV. However, our results demonstrate that this salvage pathway is not sufficient to support growth within the CCV in the absence of *de novo* NAD synthesis, indicating that the levels of nicotinic acid in the CCV are not high enough

to provide sufficient NAD to support growth or that the salvage system is not active *in vivo*. We saw similar results in both human epithelial and human macrophage-like cells, suggesting that the specific host cell does not affect the requirement of *C. burnetii* for *de novo* NAD synthesis when inside the CCV. In contrast, another intracellular human pathogen, *Mycobacterium tuberculosis*, is fully virulent in an animal model even in the absence of *de novo* NAD synthesis (25). *M. tuberculosis* possesses the Pnc salvage system, and expression of this system is induced during mouse infection (25), suggesting that, in contrast to *C. burnetii*, it is salvage pathways rather than *de novo* synthesis that are crucial for *M. tuberculosis* virulence. This also suggests that the nutrient composition of the compartment that *M. tuberculosis* replicates in is different from that of the CCV. It is also possible that the observed differences may relate to the host species used, because the *M. tuberculosis* studies were carried out in mice, whereas our study used human cells, or the observed differences may be due to differences between animal and cell infection models.

Another intracellular human pathogen, *Shigella flexneri*, lacks the *de novo* NAD synthetic pathway because of mutations within both *nadA* and *nadB* and relies on the conversion of nicotinic acid to NaMN by PncB, resulting in an absolute requirement for nicotinic acid supplementation during growth *in vitro*. In contrast to *C. burnetii*, restoring *de novo* NAD synthesis reduced the ability of *S. flexneri* to invade and spread between host cells (26). This finding appeared to relate to the inhibition of cell-to-cell spread by quinolate, although the mechanisms by which this occurs is not yet known. Nonetheless, this observation further supports the idea that tight regulation of this pathway is crucial to the virulence of intracellular pathogens. The downstream enzymes, NadD and NadE, which are common to both the salvage pathway and the *de novo* synthetic pathway, have been shown to be essential in the model organism *Mycobacterium smegmatis*, because inhibition of these enzymes leads to metabolic catastrophe and bacterial death, because of the loss of essential NAD(H) cofactors, and these enzymes are therefore considered antimycobacterial drug targets (22). In humans, three isoforms of hsNMNAT carry out the physiologically equivalent reaction to that carried out by NadD in bacteria. Although there appear to be structural distinctions between the human and prokaryotic enzymes that would make selective targeting of bacterial enzymes such as NadD possible (27), our results suggest that further investigation of the role of *de novo* NAD synthesis in a variety of bacteria may reveal that targeting the prokaryotic-specific part of this pathway is a viable alternative therapeutic target for many pathogenic bacteria.

Loss of *de novo* synthesis reduced the levels of NAD-containing metabolites within axenically cultured *C. burnetii*, demonstrating that even in rich medium the salvage pathway is not sufficient to completely overcome the loss of *de novo* synthesis, although the *nadB* mutant grew normally in axenic medium (Fig. S4). Consistent differences in the steady-state metabolic profiles of the *nadB* mutant and both WT and the complemented strain were seen in the levels of nucleotides and related metabolites, and in TCA intermediates, with lower abundances of these metabolites in the *nadB* mutant. This may suggest a

change in the flux through different key metabolic pathways when the levels of NAD-containing cofactors become limited. Furthermore, genome analysis suggests that *C. burnetii* lacks the oxidative arm of the pentose phosphate pathway, and it is not clear how NADPH, a major source of reducing equivalents for core metabolic reactions and antioxidant mechanisms, is regenerated (28). Therefore, *C. burnetii* and other bacteria that lack this pathway may be particularly vulnerable to inhibition of *de novo* NAD synthesis. We did observe some differences in changes in metabolite levels between the complemented *nadB* mutant and WT, when compared with the *nadB* mutant, which most likely relates to the overexpression of *nadB* in the complemented strain caused by the use of a plasmid for genetic complementation. Future studies employing stable isotope labeling would further define how the reduced levels of NAD-containing metabolites affect the overall cellular metabolism of *C. burnetii*.

Adequate levels of NAD-containing cofactors are crucial for cellular metabolism and maintenance of redox homeostasis. Furthermore, given the highly oxidative environment of the phagolysosome, it is expected that genes associated with redox balance are important to *C. burnetii* intracellular survival and replication. Here, we demonstrate conclusively that *de novo* NAD synthesis is critical for *C. burnetii* pathogenesis and suggest that further investigation of the importance of this prokaryotic-specific pathway in the virulence of a wider range of pathogens is warranted.

Experimental procedures

Cell strains and culture conditions

Plaque purified *C. burnetii* Nine Mile phase II (NMII) strain RSA 439 and derivatives were cultured in ACCM-2 liquid medium or ACCM-2-agarose plates at 37 °C with 5% CO₂ and 2.5% O₂. Chloramphenicol and kanamycin at final concentrations of 3 and 350 µg/ml, respectively, were used for selection of transposons and plasmids when required. *E. coli* JM109 was used for expression and purification of recombinant proteins and *E. coli* DH5a for routine cloning. *E. coli* XL1-Blue super competent cells were used for site-directed mutagenesis. *E. coli* was cultured in Luria–Bertani medium with ampicillin (100 µg/ml), chloramphenicol (25 µg/ml), and kanamycin (100 µg/ml) added as required for plasmid selection. HeLa CCL2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 5 or 10% fetal calf serum (FCS) at 37 °C in 5% CO₂.

Clonal isolation and complementation of the *nadB* mutants

The *C. burnetii* *nadB* transposon mutants from the previous screen (12) were clonally isolated as previously described (13). Briefly, clones were isolated by growth on two-layered ACCM-2 containing chloramphenicol. The plates were incubated for 6 days before single colonies were harvested using a sterile micropipette tip and expanded into 24-well plates. DNA was extracted from axenically cultured *C. burnetii* using the High Pure PCR template preparation kit as per the manufacturer's instructions (Roche). The cultures were screened by PCR using gene-specific and transposon primers to confirm the presence of the transposon within the *nadB* ORF.

Table 1
Primers used in this study

Name	Sequence (5' to 3')	Annealing temperature (°C)	Purpose
<i>nadB</i> F1	AAAGTCGACATGCCACCTATGATGTCCT	62	Complementation construct
<i>nadB</i> F2	AAAGTCGACATAGCCACCTATGATGTCCT	61	Cloning into pGEX-4T-1
<i>nadB</i> R	AAAGTCGACCTAAATATGCTTTTCGCCGATT	62	Cloning into pGEX-4T-1 or complementation construct
<i>nadB</i> (Internal)	CGTCTGTAAGGGCGCAGAGG	55	Sequencing within the inserted region
<i>OmpA</i> 0008 F	CAGAGCCGGAGTCAAGCT	55	Quantification of <i>Coxiella</i> genomes
<i>OmpA</i> 0009 R	CTGAGTAGGAGATTGGAATCGC	55	Quantification of <i>Coxiella</i> genomes
pKM225 (5172) F	TGCTCACATGTTCTTTCCTGC	55	Transposon detection
pKM225 (5173) R	TGTGATGGCTTCCATGTCG	55	Transposon detection
pJB-Kan:3xFLAG F	GAGCTGTTGACATTAATCATC	55	Sequencing complementation construct
pJB-Kan:3xFLAG R	GGATTCATCGACTGTGGCCG	55	Sequencing complementation construct
T7	TAATACGACTCACTA	55	Sequencing
SP6	TATTTAGGTGACACT	55	Sequencing
pGEX-4T-1 F	GGGCTGGCAAGCCACGTTTGGTG	55	Sequencing pGEX- <i>nadB</i> construct
pGEX-4T-1 R	CCGGGAGCTGCATGTGTCAAGAGG	55	Sequencing pGEX- <i>nadB</i> construct
R275L F	GGAGATGGCCCTCTAGACATTGTGCTC	55	Site-directed mutagenesis
R275L R	GAGCGACAATGTCTAGAGGGCCATCTCC	55	Site-directed mutagenesis

To complement the *nadB* mutant, primers (Table 1) were used to amplify *nadB* with engineered *Sall* restriction sites to facilitate cloning into the *C. burnetii* complementation vector pJB-Kan:3xFLAG (29) to produce pFLAG-NadB. DNA sequencing was used to confirm the insertion of full-length *nadB* in the correct orientation. pFLAG-NadB was then introduced into the *C. burnetii nadB* mutant as described previously (12). Briefly, axenically cultured stationary phase *C. burnetii* were harvested by centrifugation ($15,000 \times g$, 4 °C, 15 min) and resuspended in 20 ml of ice-cold 10% glycerol, before cells were harvested again and resuspended in 100 μ l of ice-cold 10% glycerol. 10 μ g of plasmid was then introduced via electroporation (18 kV, 500 Ω , and 25 microfarads). Following overnight recovery, kanamycin (350 μ g/ml) was added, and transformants were selected on ACCM-2–agarose plates. The colonies were expanded to 24-well plates in ACCM-2 and screened for expression of FLAG-NadB using SDS-PAGE immunoblotting. Mouse anti-FLAG antibody was diluted (1/1000) in 1% (w/v) skim milk in PBS-T, and the secondary antibody (anti-mouse IgG HRP) was diluted to 1/3000 in 1% (w/v) skim milk in PBS-T for immunoblotting, before detection of signal using chemiluminescence.

Intracellular growth curves and immunofluorescence microscopy

The day prior to infection, 5×10^4 HeLa CCL2 cells were seeded into 24-well plates with or without coverslips. Stationary phase axenically grown *C. burnetii* strains were resuspended in DMEM + 5% FCS, and bacterial numbers were quantified by qPCR using *ompA*-specific primers (30). Bacteria were then diluted in DMEM + 5% FCS at 1×10^7 genome equivalents/ml to achieve a multiplicity of infection of 50. HeLa cells were infected for 4 h, and then the medium was removed, washed with PBS to remove uninfected bacteria, and incubated with fresh DMEM with 5% FBS. Infection lysate was collected at the time of infection (day 0) and 24 (day 1), 72 (day 3), 120 (day 5), and 168 (day 7) h after this initial time point. At each time point, the cells were lysed with H_2O , samples were harvested, and the *C. burnetii* genome equivalents were determined using *ompA*-specific qPCR.

Replicate wells were also fixed with 4% paraformaldehyde at day 3 for subsequent immunofluorescent staining. The fixed

cells were permeabilized and blocked with blocking buffer (PBS + 2% (w/v) BSA + 0.05% (v/v) saponin) for 1 h. The cells were then stained with rabbit anti-*C. burnetii* and mouse anti-LAMP1 monoclonal antibodies diluted in blocking buffer at 1/10,000 and 1/500, respectively. Secondary antibodies anti-rabbit Alexa Fluor 568 and anti-mouse 488 were used diluted at 1/3000 in blocking buffer. DAPI diluted 1/10,000 in PBS was added following removal of the secondary antibodies, and the cells washed were with PBS before mounting on glass slides using Dako fluorescent mounting medium. The images were acquired using Zeiss LSM700, Zeiss LSM710, and Nikon A1R confocal microscopes and analyzed using ImageJ software.

Three days prior to infection, THP-1 cells were seeded at 5×10^5 /well in 24 well plates and treated with 10 nM phorbol 12-myristate 13-acetate to induce differentiation into macrophage-like cells. THP-1 cells were then infected at a multiplicity of infection of 25, and the intracellular replication assays were performed in a similar manner as for the HeLa cells. To confirm expression of FLAG-tagged proteins by *C. burnetii* strains during THP-1 infection, infected THP-1 cells were harvested 3 days after infection and subjected to SDS-PAGE. Mouse anti-FLAG antibody was diluted (1/1000) in 1% (w/v) skim milk in PBS-T and the secondary antibody (anti-mouse IgG HRP) diluted to 1/2000 in 1% (w/v) skim milk in PBS-T for immunoblotting, before detection of signal using chemiluminescence.

Site-directed mutagenesis

Sequence alignment using Geneious software was performed to identify conserved amino acids between the *E. coli* NadB protein (NP_417069.1, b2574) and *C. burnetii* NadB. *C. burnetii nadB* was amplified using gene-specific primers (Table 1) and cloned into pGEM-T (Promega) as per the manufacturer's instructions. To mutate Arg²⁷⁵ to Leu, primers were designed to introduce the desired mutation (Table 1) and site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Agilent Technologies) carried out following the manufacturer's instructions. The presence of the desired mutation in pGEM-R275L-NadB was verified by DNA sequencing. This plasmid was then used to subclone R275L-NadB into the *C. burnetii* complementation vector pJB-Kan:3xFLAG to produce pFLAG-R275L-NadB and as template for PCR amplifica-

nadB is required for *C. burnetii* pathogenesis

tion of R257L-*nadB* for cloning into pGEX-4T-1 to produce GST-R257L-NadB. The pFLAG-R257L-NadB construct was introduced into the *C. burnetii nadB* mutant as described above, with expression of full-length R257L-NadB confirmed by anti-FLAG immunoblotting, as for the original complementation construct (Fig. S1).

Expression and purification of recombinant proteins

Both *nadB* and R257L-NadB were PCR-amplified using gene-specific primers with engineered Sall sites (Table 1) and cloned into the Sall site of pGEX-4T-1 with an N-terminal GST tag. Correct insertion of the gene was confirmed by DNA sequencing. To purify recombinant proteins, overnight cultures of *E. coli* JM109 were diluted 1 in 100 in LB broth and grown to late logarithmic phase before induction of protein expression using 1 mM isopropyl- β -D-thiogalactopyranoside. Bacteria were harvested by centrifugation ($7700 \times g$, 10 min, 4°C) and incubated overnight in ice-cold PBS containing 1 mg/ml lysozyme and 1 mM phenylmethylsulfonyl fluoride. The cells were lysed by sonication, Triton X-100 was added to a final concentration of 1%, and the lysate was incubated for 30 min on ice. The soluble and insoluble fractions were separated by centrifugation. Recombinant proteins were purified from the soluble fraction using affinity chromatography on a GSH-Sephacrose column according to the manufacturer's instructions (Amersham Pharmacia Biotech). Fractions containing the desired protein were pooled, and the purified protein was dialyzed against PBS using dialysis tubing cellulose membrane (Sigma) and protein concentration determined by Pierce BCA protein assay kit (Thermo Scientific) using BSA standards.

Determination of L-aspartate oxidase activity

A two-step enzyme assay was conducted to determine the L-aspartate oxidase activity of the recombinant proteins. In the first step, 100 mM bicine, pH 8.0, 2 μM FAD, 10 mM L-aspartate, 0.5 mg of BSA, 50 units of catalase, and 4.9 μg of recombinant protein (GST-NadB, GST-R257L-NadB, or GST) was incubated at 37°C for 20 min and then stopped with perchloric acid. The denatured protein was removed by centrifugation ($1000 \times g$, 3 min, RT). The supernatant was transferred to a new tube and neutralized with potassium hydroxide. For the second step, 0.1 mM NADH, 2.5 mM EDTA, and 100 mM bicine were added to the supernatant, and the reaction was started with 10 units of malate dehydrogenase (Sigma). The absorbance at 340 nm was measured at 1-min intervals for 8 min at 25°C , and the average optical density of triplicate wells was calculated at each time point (following subtraction of the blank absorbance reading).

Quenching and extraction of polar metabolites

Six biological replicates of 20 ml of axenically cultured *C. burnetii* NMII strains were transferred into 50 ml of prechilled falcon tubes and immediately quenched to 0°C in an ethanol–dry ice bath to stop metabolism. The quenched cultures were harvested by centrifugation (0°C , $1000 \times g$, 20 min). To remove residual culture medium, the cell pellets were washed with ice-cold PBS (0°C at $1000 \times g$, 20 min), resuspended in 1 ml of PBS, and transferred into prechilled 1.5-

ml microcentrifuge tubes. Following centrifugation (0°C at $23,000 \times g$, 15 min), the pellet was stored on ice. To lyse cells and extract metabolites, 400 μl of 3:1 (v/v) methanol:water containing 1 nmol of $^{13}\text{C}_6$ -sorbitol and 10 nmol of $^{13}\text{C}_5$, ^{15}N -labeled valine as internal standards was added. Following vigorous mixing the cells were lysed using freeze-thaw cycles, and the reaction mixture was adjusted to a CHCl_3 : CH_3OH : H_2O ratio of 1:3:1 (v/v/v) by the addition of chloroform before mixing. After a 10-min incubation period, centrifugation (0°C at $23,000 \times g$ for 15 min) was performed to pellet cell debris and precipitated proteins. Phase separation was achieved after the supernatant was transferred to a fresh microcentrifuge tube and adjusted to CHCl_3 : CH_3OH : H_2O ratio of 1:3:3 (v/v/v) by addition of water. The upper aqueous phase containing polar metabolites was transferred to a fresh microcentrifuge tube and stored at -80°C until drying. The samples were dried under vacuum at 37°C with 30 μl of methanol added to the dried sample for the final drying stage. Polar metabolites were then derivatized and analyzed by GC/MS using a Shimadzu GC-TQ8040 triple quadrupole, as described previously (31, 32). Polar metabolites were also analyzed by HPLC–MS using an Agilent Technologies 1200 series HPLC system coupled to a 6545 Q-TOF, as described previously (31, 32).

Statistical analysis and comparison of metabolite profiles

Metabolites from all strains were identified from representative chromatograms by using an Agilent MSD Productivity Chemstation. Metabolites were identified by comparison of retention times and molecular masses with authentic standards. Peak integration was performed on the spectra from identified metabolites and collated into a targeted data matrix. The data were then missing value-imputed, median-normalized, and log-transformed and statistically analyzed by R-script using R-based statistical analysis package from Metabolomics Australia, as described previously (32) and the online tool MetaboAnalyst. Metabolite abundances between selected strain pairs were compared using unpaired Student's *t* test with the Benjamini–Hochberg adjustment to correct for false discoveries, with $p < 0.05$ considered significant.

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***De novo* NAD synthesis is required for intracellular replication of *Coxiella burnetii*,
the causative agent of the neglected zoonotic disease Q fever**

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Chapter 3. Meso-tartrate inhibits intracellular replication of *Coxiella burnetii*, the causative agent of the zoonotic disease Q fever

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RESEARCH ARTICLE

Meso-tartrate inhibits intracellular replication of *Coxiella burnetii*, the causative agent of the zoonotic disease Q fever

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One sentence summary: The growth of the disease-causing bacterium *Coxiella burnetii* inside human cells can be significantly decreased by using a compound non-toxic to human cells.

Editor: Andrew Olive

ABSTRACT

The zoonotic disease Q fever caused by the intracellular bacterium *Coxiella burnetii* remains a global health threat due to its high infectivity, environmental stability, the debilitating nature and the long duration of treatment. Designing new and potent drugs that target previously unexplored pathways is essential to shorten treatment time and minimise antibiotic resistance. Nicotinamide adenine dinucleotide (NAD) is an essential and ubiquitous cofactor in all living organisms. NadB, an L-aspartate oxidase catalysing the first step of the prokaryotic-specific NAD *de novo* biosynthetic pathway, is required for *C. burnetii* growth and replication inside host cells. In this study, *in vitro* enzyme assays utilising recombinant glutathione S-transferase tagged NadB (GST-NadB) demonstrated inhibition of the L-aspartate oxidase activity of NadB by meso-tartrate. Furthermore, meso-tartrate inhibits intracellular growth and replication of *C. burnetii* inside host cells in a dose-dependent manner, and has no effect on the viability of mammalian cells. Unexpectedly, meso-tartrate also inhibited growth of *C. burnetii* in axenic medium, and further reduces replication of the *nadB* mutant inside host cells, suggesting it is acting more widely than simple inhibition of NadB. Overall, these results suggest that the antibacterial activity of meso-tartrate warrants further study, including investigation of its additional target(s).

Keywords: Q fever; *Coxiella burnetii*; antimicrobial; meso-tartrate; NAD synthesis; enzyme inhibition

INTRODUCTION

Coxiella burnetii, an intracellular Gram-negative pathogenic bacterium, is the causative agent of Q fever in humans, a disease that presents in both acute and chronic forms and which is

transmitted primarily by inhalation of contaminated aerosols. Disease in humans is usually acquired from animals, with ruminant livestock a major reservoir (Moffatt, Newton and Newton 2015). *Coxiella burnetii* is a pleomorphic organism with a biphasic life cycle comprised of the replicating large cell variant

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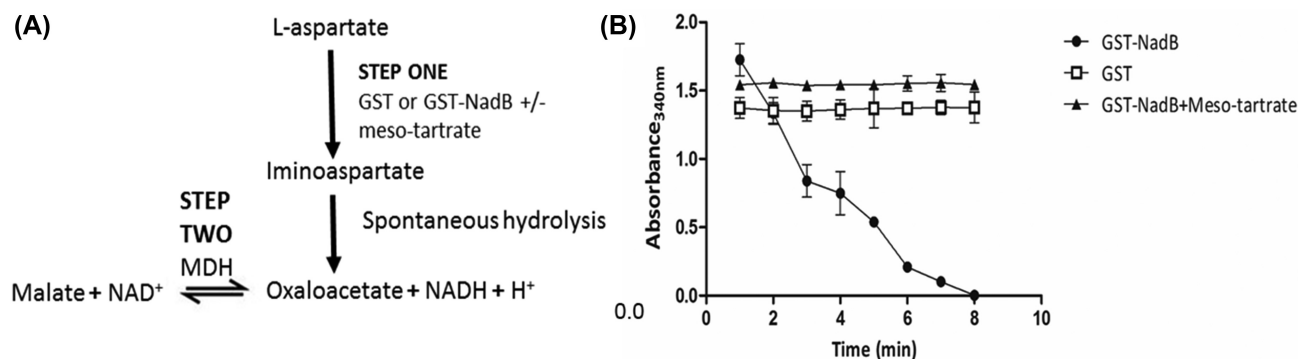


Figure 1. (A) Schematic of the two-step *in vitro* enzyme assay. In the first step of the *in vitro* assay, NadB converts L-aspartate to iminoaspartate, which then spontaneously hydrolyses to OAA. In the second step of the assay, commercial MDH was used to convert this OAA to malate, to measure if iminoaspartate was produced in step one. Conversion of OAA to malate is monitored by measuring the oxidation of the co-factor NADH to NAD⁺ by measuring the change in absorbance at 340 nm. A fall in absorbance in the second step therefore indicates that NadB was active in the first step. (B) Meso-tartrate inhibits enzymatic activity of NadB. The change in absorbance at 340 nm when the conditions used in the first step were GST-NadB (closed circles), GST (open squares) and GST-NadB + 200 mM meso-tartrate (triangles), demonstrating that no OAA was present in the second step for GST-NadB + 200 mM meso-tartrate, and that meso-tartrate therefore inhibits NadB. The graph represents the mean $\pm$ SD of three independent experiments.

form and the environmentally stable but non-replicating small cell variant form (Shaw and Voth 2019). In a natural mammalian infection, *C. burnetii* has a high affinity for alveolar macrophages and once internalised replicates inside the *Coxiella*-containing vacuole (CCV), an acidic and highly oxidative vacuole containing active lysosomal proteases and lysosomal enzymes, including cathepsin D (van Schaik *et al.* 2013).

In order to survive and replicate inside the host cell, *C. burnetii* requires a functional Dot/Icm system (Beare *et al.* 2011; Carey *et al.* 2011), a type IV secretion system that translocates at least 130 effector proteins into the host cell to control various infection events (Moffatt, Newton and Newton 2015). These effector proteins are required for inhibition of apoptosis (Lührmann *et al.* 2010), stimulation of autophagy (Martinez *et al.* 2016; Latomanski and Newton 2018) and formation of the spacious replicative CCV (Larson *et al.* 2013; Weber *et al.* 2013; Newton *et al.* 2014; Latomanski *et al.* 2016; Crabill *et al.* 2018), and are the focus of many studies on *C. burnetii* molecular pathogenesis. However, intracellular proteins may also play crucial roles, and we recently demonstrated that *C. burnetii* NadB is an L-aspartate oxidase required for *de novo* nicotinamide adenine dinucleotide (NAD) synthesis, and that NadB is required for normal CCV biogenesis and *C. burnetii* intracellular replication (Bitew *et al.* 2018).

NAD is an essential molecule in all living organisms and is involved in numerous biological and core metabolic reactions (Gazzaniga *et al.* 2009; Osterman 2009). Maintenance of correct intracellular NAD⁺/NADH ratio is vital for survival (Mesquita *et al.* 2016). NadB is part of the prokaryotic-specific NAD biosynthetic pathway, and therefore an attractive potential therapeutic target. Meso-tartrate is a form of tartaric acid, which naturally occurs in numerous fruits, predominantly in grapes, but also in citrus, bananas and tamarinds (Duarte *et al.* 2012). It was previously shown to inhibit the activity of *Escherichia coli* L-aspartate oxidase (Nasu, Wicks and Gholson 1982). In this current study, *in vitro* enzyme assays demonstrated that meso-tartrate inhibits the L-aspartate oxidase activity of NadB in a dose-dependent manner. Furthermore, meso-tartrate inhibits *C. burnetii* replication inside both epithelial and macrophage-like mammalian cells, but has no effect on the viability of these mammalian cells. Unexpectedly, meso-tartrate also inhibited the growth of the *nadB* transposon mutant inside host cells in a dose-dependent manner, indicating that meso-tartrate is acting on at least one additional bacterial target.

RESULTS

Meso-tartrate inhibits the L-aspartate oxidase activity of NadB *in vitro*

Previously, recombinant GST-NadB was purified and demonstrated to have L-aspartate oxidase activity *in vitro* (Bitew *et al.* 2018) using a two-step assay (Fig. 1A). In the second step of this assay, oxaloacetate (OAA), produced by spontaneous hydrolysis of the iminoaspartate produced by NadB in the first step, is converted to malate by commercial malate dehydrogenase (MDH). MDH requires the cofactor NADH, and concurrent oxidation of NADH to NAD⁺ can be measured by monitoring the change in absorbance at 340 nm. Absorbance decreases in the second step only when active NadB in the first step produces iminoaspartate (Bitew *et al.* 2018).

To investigate whether meso-tartrate inhibits enzymatic activity of NadB, the two-step assay was carried out with 200 mM of meso-tartrate added to the first step of the reaction. Similar to the GST control, no change in absorbance was observed in the second step, indicating that no iminoaspartate was produced in the first step and that meso-tartrate inhibits the L-aspartate oxidase activity of NadB (Fig. 1B).

Meso-tartrate inhibits intracellular replication of *C. burnetii* in both epithelial and macrophage-like cells

Enzymatically active NadB is required for intracellular replication of *C. burnetii* (Bitew *et al.* 2018). To investigate if meso-tartrate could inhibit NadB during infection of mammalian cells, and therefore reduce *C. burnetii* intracellular replication, HeLa cells and THP-1 cells were infected with *C. burnetii* and cultivated in medium containing 200 mM meso-tartrate. Replication of *C. burnetii* over 7 days was monitored using quantitative polymerase chain reaction (qPCR). *Coxiella burnetii* grown in HeLa cells cultivated in medium supplemented with meso-tartrate displayed significantly reduced bacterial replication compared to *C. burnetii* replication in control HeLa cells cultured in normal medium. Only a 50-fold increase in genome equivalents at 7 days post-infection occurred in the meso-tartrate positive conditions, whereas in the absence of meso-tartrate *C. burnetii* showed over 400-fold increase in genome equivalents at 7 days post-infection (Fig. 2A). The growth over 7 days in the presence

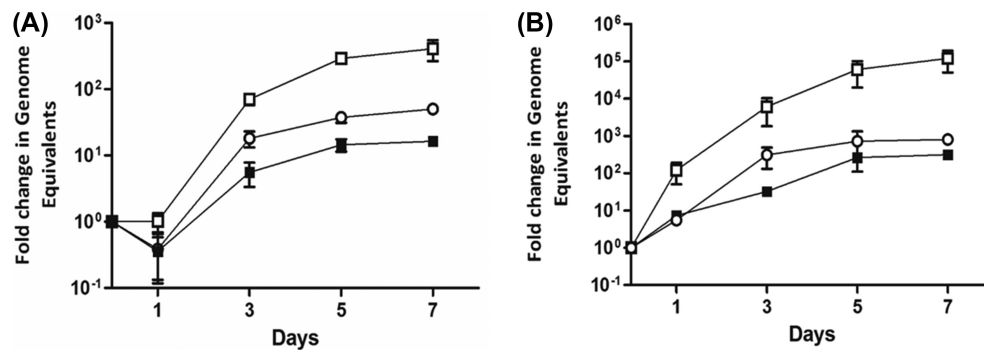


Figure 2. Meso-tartrate inhibits intracellular replication of *C. burnetii*. Intracellular growth of *C. burnetii* NMII (open squares), *C. burnetii* NMII + meso-tartrate (open circles) and *nadB::Tn* (closed squares) at days 1, 3, 5 and 7 post-infection in HeLa epithelial cells (A) and THP-1 macrophage-like cells (B). The fold change in genome equivalents relative to day 0 of each strain was determined by qPCR using *ompA* specific primers. Both *nadB::Tn* and wild-type *C. burnetii* + meso-tartrate showed a statistically significant decrease in fold change in genome equivalents when individually compared to *C. burnetii* – meso-tartrate at days 3, 5 and 7 ($P < 0.05$, paired t-tests), demonstrating the inhibitory effect of 200 mM meso-tartrate in the host cell medium on *C. burnetii* growth and replication. This result represents three independent experiments that are displayed as mean $\pm$ standard deviation of three independent infections.

of meso-tartrate was more similar to that of the *nadB* mutant. In a similar way, *C. burnetii* replicating within THP-1 cells cultured in medium supplemented with 200 mM meso-tartrate were significantly reduced in replication over 7 days, with only an 802-fold increase in genome equivalents at day 7 compared with a 1.2×10^5 fold increase in wild-type *C. burnetii* grown in THP-1 cells cultured in meso-tartrate negative medium (Fig. 2B). Replication of *C. burnetii* in THP-1 cells under meso-tartrate positive conditions was similar to that of the *nadB* mutant.

Qualitative analysis using fluorescence microscopy demonstrated that *C. burnetii* replicating in both mammalian cell lines in the presence of meso-tartrate displayed a smaller CCV containing fewer bacteria (Fig. 3A and B), similar to the *nadB* mutant, whereas *C. burnetii* grown in meso-tartrate negative conditions formed a much bigger vacuole containing many bacteria (Fig. 3A and B).

Meso-tartrate is not cytotoxic for HeLa or THP-1 cells

To investigate if the reduction in intracellular growth was due to a toxic effect of meso-tartrate on the mammalian cells themselves, a cytotoxicity assay was conducted for both HeLa and THP-1 cells. As the intracellular replication assays were carried out over 7 days, viability assays were performed at 24 h and 7 days after the addition of meso-tartrate to the mammalian cell culture medium. Meso-tartrate at concentrations of 50, 100, 150 and 200 mM did not significantly reduce the cellular viability of HeLa cells and THP-1 cells at 24 h and 7 days compared to untreated cells, as measured by the number of active metabolic cells present under each condition using a commercial cell viability kit (Fig. 4) that utilises soluble tetrazolium salt WST-8, which is reduced by cellular dehydrogenase present in viable cells into yellow coloured product (formazan). The formation of formazan dye is directly proportional to the number of living cells.

Meso-tartrate inhibits *C. burnetii* replication inside THP-1 cells in a dose-dependent manner

To confirm the inhibition of *C. burnetii* growth inside macrophage-like cells by meso-tartrate, a dose-response analysis was carried out in THP-1 cells. At day 7, all four concentrations of meso-tartrate tested (50, 100, 150 and 200 mM) significantly reduced *C. burnetii* compared to the untreated

control. Furthermore, the growth inhibition induced by 200 mM meso-tartrate was significantly higher than that induced by 50 or 100 mM, demonstrating that the observed inhibition was dose dependent (Fig. 5A).

Meso-tartrate also inhibits replication of the *nadB* transposon mutant inside THP-1 cells, indicating that it acts on additional bacterial target(s)

Similar to its effect on *C. burnetii* NMII, meso-tartrate was, surprisingly, able to inhibit replication of the *nadB::Tn* mutant inside THP-1 cells in a dose-dependent manner (Fig. 5B). This suggests that meso-tartrate is acting on at least one other *C. burnetii* target to reduce intracellular replication of the bacterium. Similar inhibition was also observed for the complemented strain *nadB::Tn* pFLAG:*nadB* (Fig. 5C).

Meso-tartrate inhibits the growth of *C. burnetii* in vitro

Although enzymatically active NadB is required for optimal replication inside host cells, in the host-cell free medium ACCM-2 the *C. burnetii* *nadB* mutant replicates normally, demonstrating that active NadB is not required *in vitro* (Bitew et al. 2018). Further supporting the presence of additional targets for meso-tartrate, replication of *C. burnetii* in ACCM-2 was significantly inhibited by addition of 50, 100 and 150 mM of meso-tartrate, with a reduction in genome equivalents at day 7 when compared to the untreated *C. burnetii* control (Fig. 6B). In addition, replication of the *nadB::Tn* mutant was also inhibited in axenic medium, as was replication of the complemented strain *nadB::Tn* pFLAG:*nadB* (Fig. 5B and C), suggesting that meso-tartrate is able to inhibit other crucial processes.

DISCUSSION

Disrupting the NAD *de novo* biosynthesis pathway in *C. burnetii* results in significantly reduced replication inside host cells (Bitew et al. 2018), suggesting that targeting this prokaryotic specific pathway may result in novel therapeutics. Recent studies demonstrated that targeting NadD and NadE, enzymes downstream of NadB in the NAD *de novo* biosynthesis pathway, may lead to the production of highly effective drugs against the important human pathogen *Mycobacterium tuberculosis* (Rodionova et al. 2014; Osterman et al. 2019). In the current

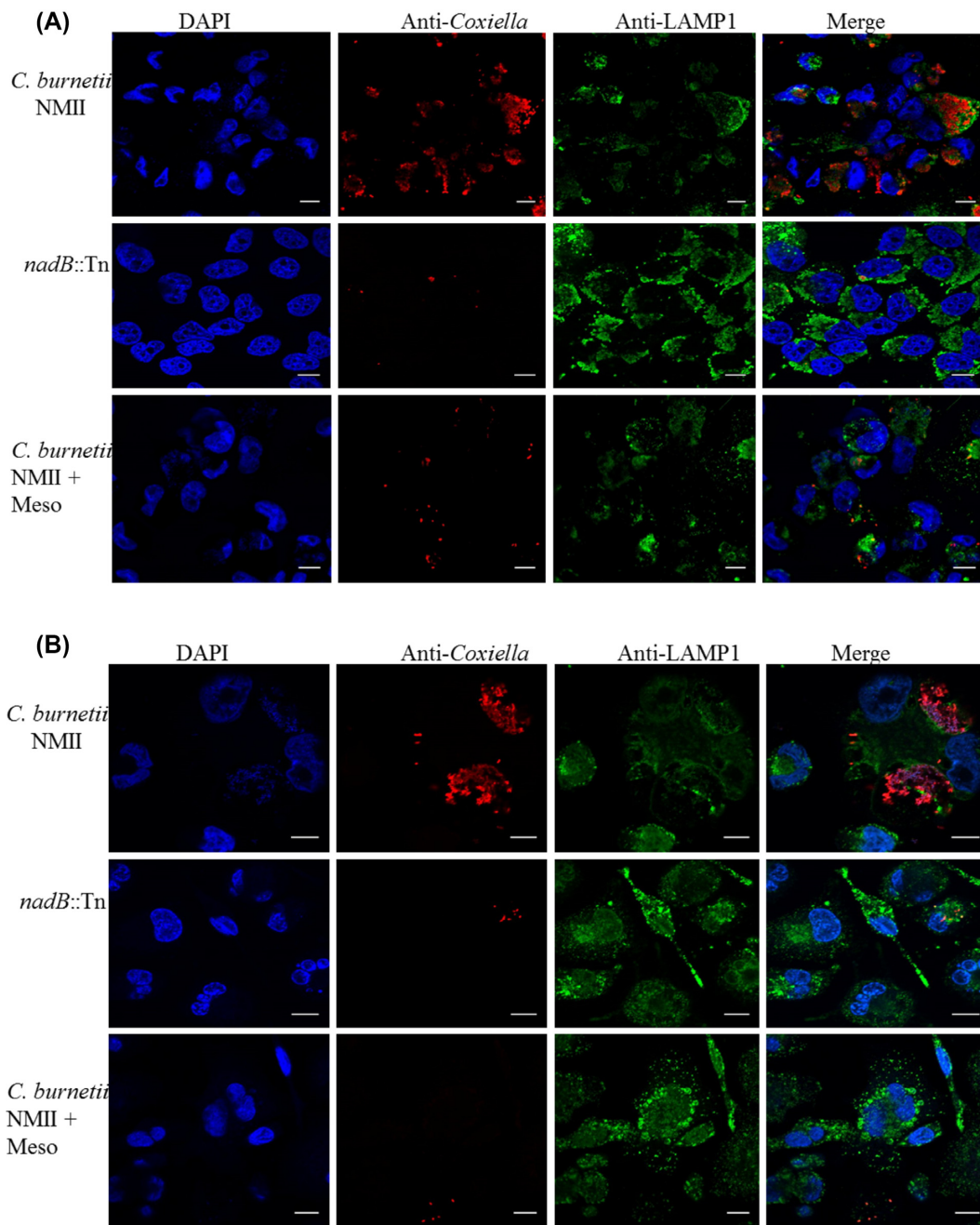


Figure 3. Representative micrographs demonstrating that meso-tartrate treatment of host cells results in smaller CCVs and fewer bacteria inside host cells. HeLa cells (A) and THP-1 cells (B) were infected with *C. burnetii* strains at multiplicity of infection (MOI) of 50 and 25, respectively. Three days post-infection the cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS). The cells were stained with rabbit anti-*Coxiella* antibody (red) and mouse anti-LAMP1 antibody (green) in blocking buffer and DNA stained with DAPI (blue) diluted in PBS. Both the *nadB* mutant and wild-type *C. burnetii* + meso-tartrate produced smaller vacuoles containing fewer bacteria, whereas wild-type *C. burnetii* – meso tartrate formed much bigger vacuoles containing a large number of bacteria. Image acquisition was conducted by Nikon A1R confocal microscope and the images are representative of three independent experiments. A 10 μ m scale bar was used for these images. Meso = meso-tartrate.

study, meso-tartrate inhibited both NadB activity *in vitro* and intracellular replication of *C. burnetii* NMII, without causing cytotoxicity in host mammalian cells, suggesting it could have exciting potential as a therapeutic agent. However, and surprisingly, meso-tartrate also inhibited growth of *C. burnetii* NMII *in vitro*. NadB is not required for *C. burnetii* to replicate *in vitro*, presumably because NAD salvage pathways are able to compensate for the absence of *de novo* NAD synthesis in the rich growth medium used *in vitro* (Bitew et al. 2018). Combined with the observed

inhibition of replication of the *nadB::Tn* mutant both inside host cells and in axenic medium, this strongly indicates that meso-tartrate is targeting other prokaryotic-specific pathways. A previous historical study observed that meso-tartrate inhibited the growth of certain *Salmonella* strains *in vitro* (Old, Alfredsson and Brown 1980), which was thought to be due to inhibition of the tricarboxylic acid (TCA) cycle. As the TCA cycle is also essential in mammalian cells, this may be why the therapeutic potential of meso-tartrate as an antibacterial agent has not been

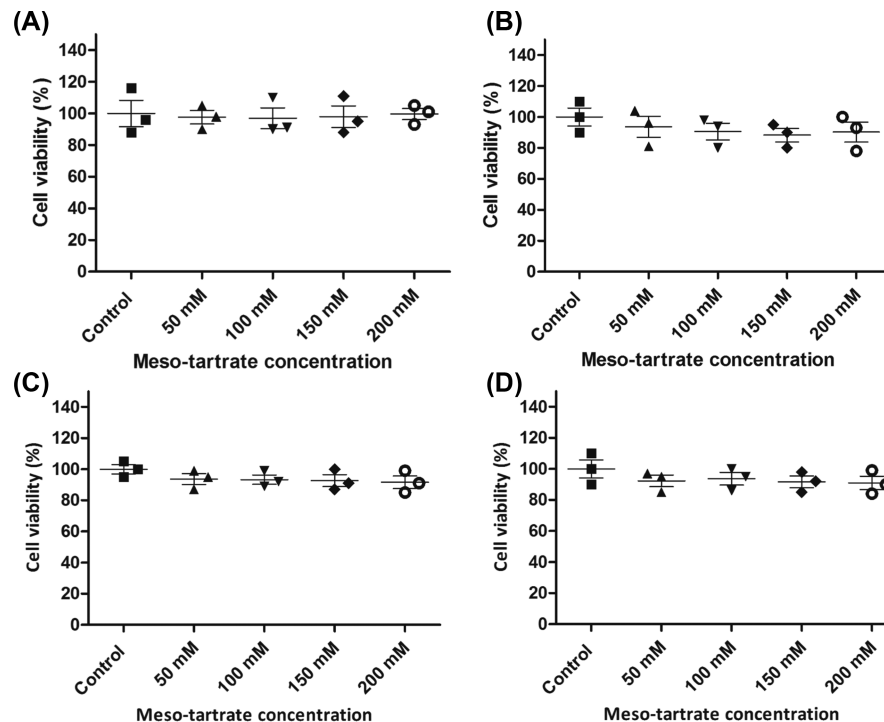


Figure 4. Cell cytotoxicity assay. Viability of HeLa cells at 24 h (A) or 7 days (B) and THP-1 cells at 24 h (C) or 7 days (D) post-treatment with meso-tartrate. Data are represented as the percentage of viable cells compared to the untreated control. Graphs represent the mean $\pm$ SD of three independent experiments, with the symbols indicating the individual mean of each biological replicate and the horizontal line the mean of all three replicates. No significant differences were observed (Student's t-test).

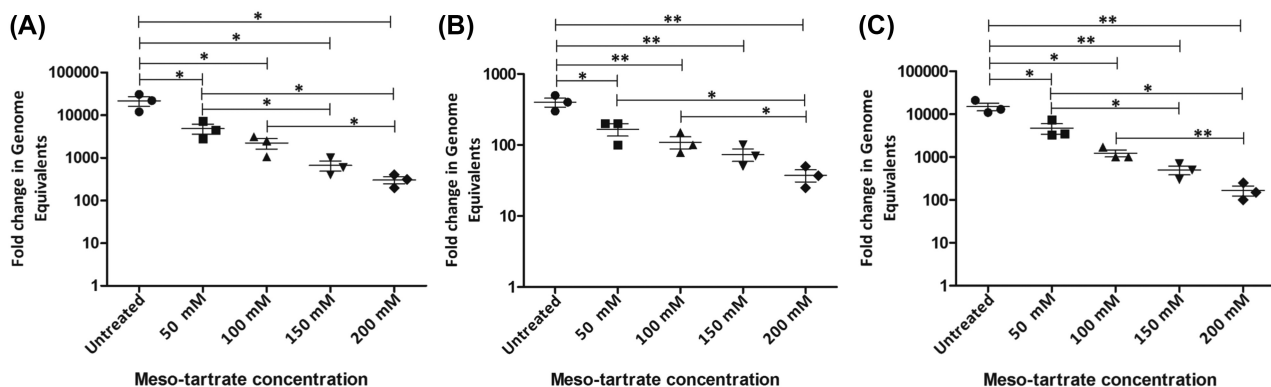


Figure 5. Meso-tartrate has a dose-dependent effect on intracellular replication of *C. burnetii*. THP-1 cells were infected with *C. burnetii* NMII (A), the *nadB::Tn* mutant (B) and the complemented strain (*nadB::Tn* pFLAG:*nadB*) (C) at MOI of 25 and treated with 50, 100, 150 and 200 mM meso-tartrate. At day 7, the infected cells were harvested and genome equivalents were determined by qPCR and are represented as to inoculum. All meso-tartrate concentrations significantly reduced the fold change in genome equivalents compared to the control, and the highest concentration tested (200 mM) significantly inhibited replication more than either 50 or 100 mM, for all three strains tested. The graphs represent the mean $\pm$ SD of three independent experiments, with the symbols indicating the individual mean of each biological replicate and the horizontal line the mean of all three replicates. * $P < 0.05$, ** $P < 0.01$ (unpaired two-tailed t-test).

previously explored. However, the lack of toxicity to mammalian cells observed in the current study suggests that the additional targets inhibited by meso-tartrate are prokaryotic-specific.

Interestingly, NadB is not considered a therapeutic target in *M. tuberculosis* as in this bacterial species the enzyme is dispensable for virulence (Rodionova et al. 2014). However, as meso-tartrate appears to be acting more broadly it may be worth investigating its effect on mycobacteria, as well as investigating if it can inhibit the replication of a number of other intracellular

pathogenic bacteria. The concentrations of meso-tartrate used in this study to achieve inhibition of replication are relatively high, suggesting that, despite its lack of toxicity on mammalian cells, in its current form meso-tartrate itself may not be a useful sole therapeutic agent. However, new compounds derived from meso-tartrate may be more potent and of use in either sole or combination therapies. Furthermore, future work identifying the other targets of meso-tartrate is likely to identify additional novel therapeutic targets.

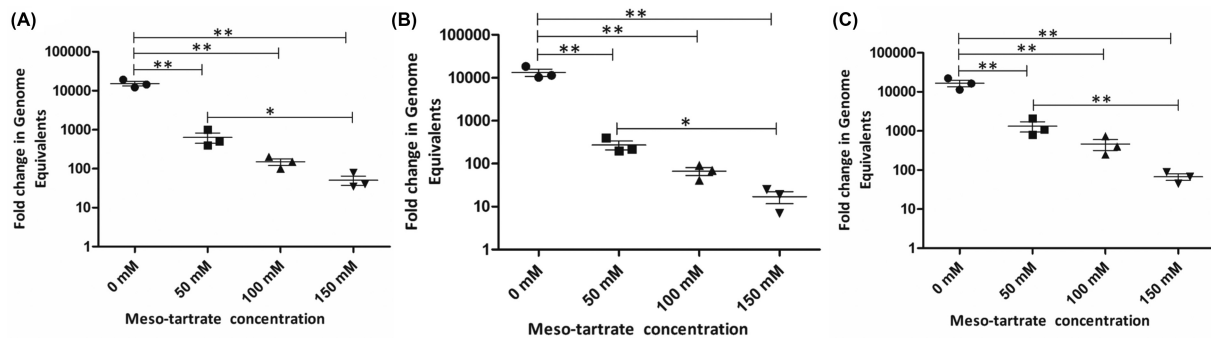


Figure 6. Meso-tartrate inhibits growth of *C. burnetii* in vitro. qPCR was used to quantify *C. burnetii* genome equivalents after 7 days of in vitro culture and the fold change in genome equivalents relative to the inoculum was determined. The growth of *C. burnetii* NMII (A), the *nadB::Tn* mutant (B) and the complemented strain (*nadB::Tn pFLAG:nadB*) (C) were all significantly inhibited by supplementing medium with 50, 100 and 150 mM of meso-tartrate. The graphs represent the mean $\pm$ SD of three independent experiments, with the symbols indicating the individual mean of each biological replicate and the horizontal line the mean of all three replicates. * $P < 0.05$, ** $P < 0.01$ (unpaired two-tailed t-test).

MATERIALS AND METHODS

Cell strains and culture conditions

Plaque purified *C. burnetii* Nine Mile phase II (NMII) strain RSA 439 and the derivative strains *nadB::Tn* and *nadB::Tn pFLAG:nadB* (Bitew et al. 2018) were cultured in liquid Acidified Citrate Cysteine Medium (ACCM-2) (Omsland et al. 2009) at 37°C with 5% CO₂ and 2.5% O₂. Chloramphenicol at a final concentrations of 3 µg/ml was used for transposon selection when culturing the *C. burnetii nadB* mutant and kanamycin (350 µg/ml) used to select for *pFLAG:nadB* (Bitew et al. 2018). Expression and purification of recombinant GST-NadB and GST was carried out using *E. coli* JM109 strain. *Escherichia coli* was cultured in Luria-Bertani medium and ampicillin (100 µg/ml) added for plasmid selection. HeLa CCL2 cells were grown and maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 5 or 10% fetal calf serum (FCS) at 37°C in 5% CO₂. THP-1 cells were grown in RPMI 1640 supplemented with 5 or 10% FCS at 37°C in 5% CO₂.

NadB inhibition assays

Recombinant GST-NadB was expressed in *E. coli* JM109 and purified as described previously (Bitew et al. 2018). The two-step enzyme assay to determine L-aspartate oxidase activity of NadB was carried out as described previously (Bitew et al. 2018). For the assays to determine the inhibitory effect of meso-tartrate, the first step of the assay consisted of 100 mM bicine, pH 8.0, 2 µM FAD, 10 mM L-aspartate, 0.5 mg bovine serum albumin and 4.9 µg of NadB, 4.9 µg GST or 4.9 µg of NadB + 200 mM meso-tartrate (Sigma). The pH of the reaction mixture was confirmed to be 8.0 after the addition of meso-tartrate, to ensure any inhibitory effects observed were not due to a change in pH. After 20 min of incubation, the reaction was stopped by adding ice cold 1.7 M perchloric acid, centrifuged (1000 $\times$ g, 3 min, RT) to remove denatured protein and the supernatant transferred to a new tube and the reaction neutralised by adding 5 M potassium hydroxide. Secondly, 0.1 mM NADH, 2.5 mM EDTA and 100 mM bicine were added to a total volume of 1 ml. The second step of the reaction was initiated by adding 10 U of malate dehydrogenase (MDH; Sigma) and absorbance at 340 nm, 25 °C was measured at 1 min interval for 8 min and the average OD calculated at each time point following subtraction of the blank absorbance reading.

Axenic growth inhibition assay

Coxiella burnetii strains were inoculated into ACCM-2 supplemented with 0, 50, 100 or 150 mM of meso-tartrate (Sigma) at 1×10^4 genome equivalents/ml and incubated for 7 days at 37°C in 5% CO₂ and 2.5% O₂. Aliquots were taken from cultures and the *C. burnetii* genome equivalents were quantified by qPCR using *ompA* specific primers, as described previously (Jaton et al. 2013). Each experiment was conducted three times and significance determined by Student's t-test relative to untreated group.

Cell viability test

The cytotoxicity of meso-tartrate was determined by measuring mammalian cell viability using a Cell Counting Kit (CCK-8) assay (Sigma). HeLa CCL2 cells were seeded in 96-well plates at a density of 1×10^4 cells per well in DMEM + 10%FCS. The cells were pre-incubated for 24 h in a humidified incubator at 37°C, 5% CO₂. Three days prior to treatment, THP-1 cells were seeded at a density of 1×10^4 cells per well in RPMI + 10%FCS in 96-well plates and treated with 10 nM phorbol 12-myristate 13-acetate to induce differentiation into macrophage-like cells. Meso-tartrate (Sigma) was dissolved in DMEM (Dulbecco's Modified Eagle Medium) or RPMI (Roswell Park Memorial Institute medium) and a final concentration of 50, 100, 150 or 200 mM of this solution was added onto cells and incubated for 24 h or 7 days. To measure viability, 10 µl of CCK-8 reagent was added to each well and the absorbance at 450 nm was measured using microplate reader following 4 h incubation. Untreated cells were used as a control and the viability of treated cells was compared to these control cells. Three independent experiments were conducted.

Intracellular replication of *C. burnetii*

Intracellular replication assays were carried out as described previously (Newton et al. 2014; Fielden et al. 2017). Briefly, 5×10^4 HeLa CCL2 cells were seeded into 24-well plates in DMEM with 10% FCS (5 trays – day 0, 1, 3, 5 and 7; strains in duplicate) the day before infection. *Coxiella burnetii* strains were grown in ACCM-2 and pelleted and resuspended in 10 ml of DMEM + 5% FCS. *Coxiella burnetii* genome equivalents were quantified by qPCR amplifying *ompA* to determine the MOI of 50, as described previously (Jaton et al. 2013). *Coxiella burnetii* diluted in DMEM + 5% FCS

or DMEM + 5% FCS supplemented with 200 mM meso-tartrate was added to each well. After 4 h of infection, the media was removed from all trays and wells and washed with PBS to remove uninfected bacteria. For day 1, day 3, day 5 and day 7 plates, fresh DMEM + 5% FCS or DMEM + 5% FCS + 200 mM meso-tartrate was added whereas for the day 0 timepoint 500 µl of distilled water was added to the wells and incubated at RT for 10 min to lyse the cells. The cells were harvested by scraping using pipette tips and the harvested lysate was pelleted and resuspended in distilled water. The *C. burnetii* genome equivalents were quantified by qPCR using *ompA*-specific primers.

THP-1 cells were seeded at 5×10^5 cells/well 72 h before infection and treated with 10 nM phorbol 12-myristate 13-acetate to induce differentiation into macrophage-like cells. The cells were infected by *C. burnetii* strains at an MOI of 25 in RPMI + 5% FCS or RPMI + 5% FCS + 200 mM meso-tartrate, and intracellular growth assays were carried out in a similar manner as performed for HeLa cells.

Immunofluorescence microscopy and observation of CCV biogenesis

Both HeLa CCL2 and THP-1 cells were seeded in 24-well plates with 10 mm glass coverslips and infected with *C. burnetii* strains in a similar way as described for the quantitative assays. After 3 days post-infection, the media were removed and the cells were fixed with 4% paraformaldehyde in PBS and blocked with PBS + 2% (w/v) BSA + 0.05% (v/v) saponin for 1 h. The cells were stained with rabbit anti-Coxiella and mouse anti-LAMP1 antibodies in blocking buffer at 1/10 000 and 1/500 dilutions, respectively. Antirabbit 568 (Coxiella) and antimouse 488 (LAMP1) diluted at 1/3000 in blocking buffer were used as a secondary antibodies. DAPI diluted at 1/10 000 in PBS was used to stain host DNA. Following staining, the coverslips were mounted on glass slides using Dako Fluorescent Mounting Medium. Nikon A1R confocal microscope was used to capture images and analysed by ImageJ.

Dose-dependent response analysis in THP-1 cells

To examine the effect of different concentrations of meso-tartrate on intracellular growth and CCV formation of *C. burnetii*, growth inhibition assays were carried out using differing concentrations of meso-tartrate. THP-1 cells were seeded in 24-well plates as described for the 7 days infection experiments and infected with *C. burnetii* strains at MOI of 25 and treated with 50, 100, 150 and 200 mM meso-tartrate in RPMI + 5%. Seven days post-infection the cells were harvested and the *C. burnetii* genome equivalents quantified by qPCR amplifying *ompA* and the fold change in genome equivalents determined relative to the inoculum. Three biological repeats were performed.

Conflict of interest. None declared.

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Chapter 4. SdrA, an NADP(H)-regenerating enzyme, is crucial for *Coxiella burnetii* to resist oxidative stress and replicate intracellularly

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RESEARCH ARTICLE

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

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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 with *C. burnetii* NMII.

KEYWORDS

Coxiella burnetii, NADP(H) metabolism, oxidative stress, short chain dehydrogenase

1 | 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, Newton, & Newton, 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, Warris, & Bleeker-Rovers, 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 4,000 human cases and the culling of thousands of animals (van Asseldonk, Prins, & Bergevoet, 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., Voth & Heinzen, 2007). The bacterium undergoes a biphasic lifecycle, differentiating from the environmentally stable small-cell variant into a replicating large cell variant 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, Newton,

Lüthmann, & Roy, 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* nicotinamide adenine dinucleotide

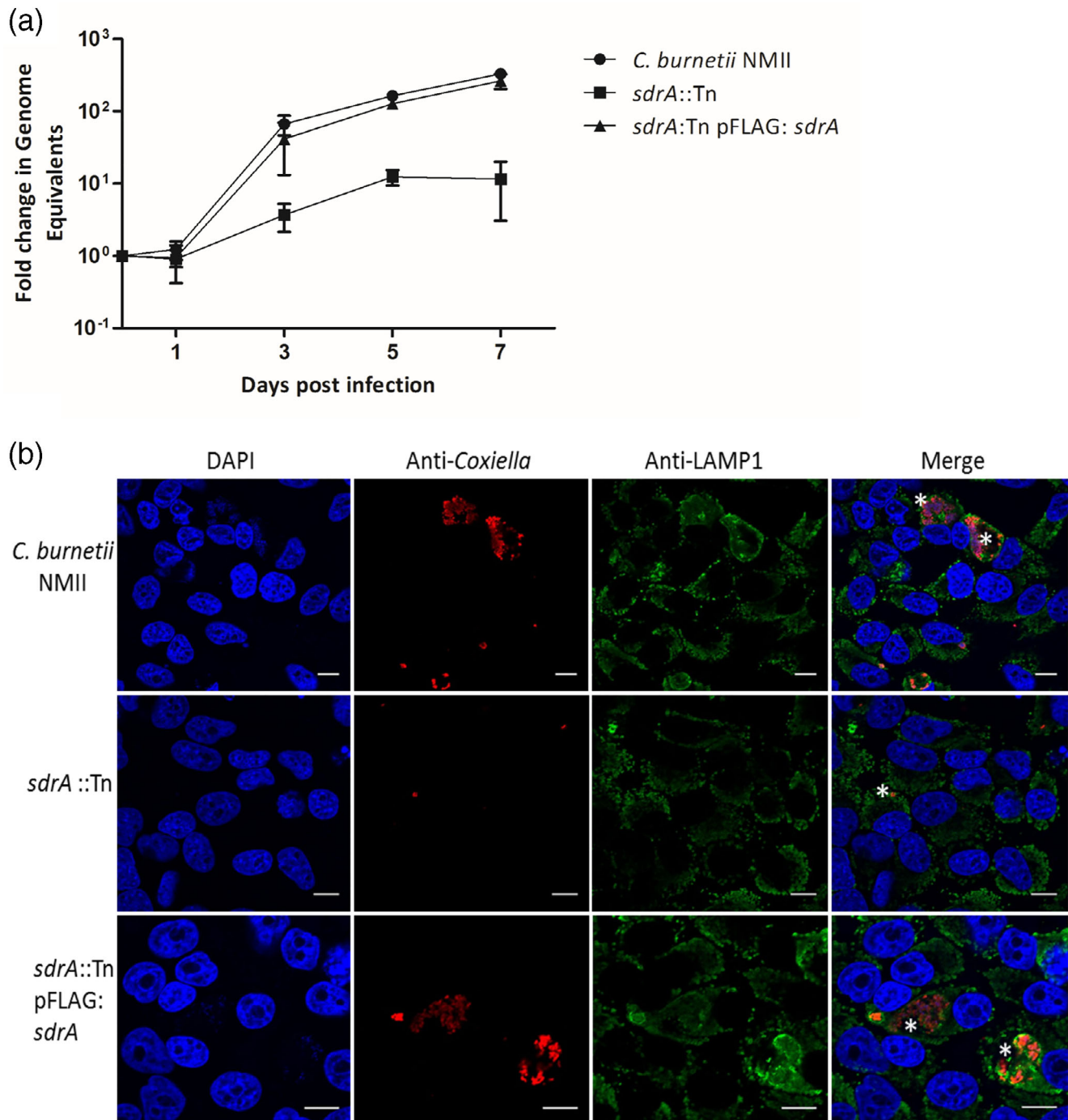


FIGURE 1 SdrA is required for intracellular replication of *C. burnetii*. (a) HeLa cells were infected by *C. burnetii* strains at a MOI of 50. At days 1, 3, 5 and 7 the fold change in genome equivalents relative to day 0 was determined by qPCR for *ompA*. The *sdrA::Tn* mutant (squares) showed a significant decrease in fold change in genome equivalents ($p < .05$, 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 $\pm$ SD (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 μ m. CCV, Coxiella-containing vacuole

(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, Lemire, Mailloux, & Appanna, 2008). *C. burnetii* lacks the oxidative branch of the pentose phosphate pathway (PPP), 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 & Heinzen, 2011). 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 (ROS) inside infected and uninfected host cells.

2 | RESULTS

2.1 | *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 a 11-fold increase in genome equivalents at 7 days post-infection, compared to *C. burnetii* Nine Mile Phase II (NMII) that showed a 330-fold increase (Figure 1a). The complemented mutant increased by 265-fold at day 7, 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).

2.2 | *SdrA* reduces NADP⁺ to NADP(H) *in vitro*

To investigate if *SdrA* can convert NADP⁺ to NADP(H), recombinant 6xHis-*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, Ying, Yang, & Wang, 2011), was utilised in the assay with NADP⁺ as the cofactor. Following the addition of recombinant enzyme, an increase in absorbance at 340 nm 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 6xHis-*SdrA* in the assay mixture.

2.3 | 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

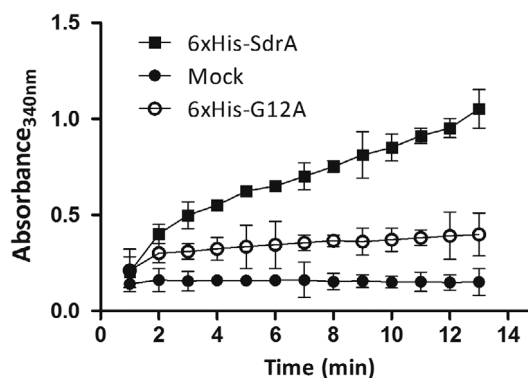


FIGURE 2 *SdrA* facilitates reduction of NADP to NADP(H) and substituting glycine-12 with alanine results in minimal NADP(H) synthesis. A change in absorbance at 340 nm occurs if NADP⁺ is reduced to NADP(H), with concurrent oxidation of ethyl (S)-(-)-4-chloro-3-hydroxybutyrate. A significant increase 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 ($p < .05$, 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* ($p < .05$, paired t test) from the 4 min timepoint onwards, indicating minimal residual enzymatic activity. Squares indicate recombinant 6xHis-*SdrA*, open circles represent 6xHis-G12A, whereas closed circles are mock purified proteins, which serve as the negative control. Error bars represent mean $\pm$ SD ($n = 3$)

(Hua, Wu, Sargsyan, & Lim, 2014), was mutated to alanine. Recombinant 6xHis-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 6xHis-G12A was used in the *in vitro* enzyme assay, minimal change in absorbance at 340 nm 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 7 (Figure 3a), compared to *C. burnetii* NMII that increased by 6.2×10^5 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 increase in genome equivalents at day 7. 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*.

2.4 | 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 H₂O₂ for 7 days before the genome equivalents were quantified by qPCR and compared to untreated controls, as an indirect measure of survival of the H₂O₂ exposure at day 0 (as this compound is inherently unstable and breaks down in culture medium rapidly). At a low concentration (50 μ M) of H₂O₂, no strains were susceptible to oxidative stress, with no significant difference in replication between untreated and treated cultures. At 500 μ M H₂O₂ *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 7. In contrast, treatment of *sdrA::Tn* with 500 μ M H₂O₂ resulted in a significant reduction in replication at day 7 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 (1 mM H₂O₂) resulted in a statistically significant decrease in

genome equivalents of all strains at day 7 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*. These 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).

2.5 | 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 7, 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.

2.6 | 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, Weusthuis, Van Der Oost, & Kengen, 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 were 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

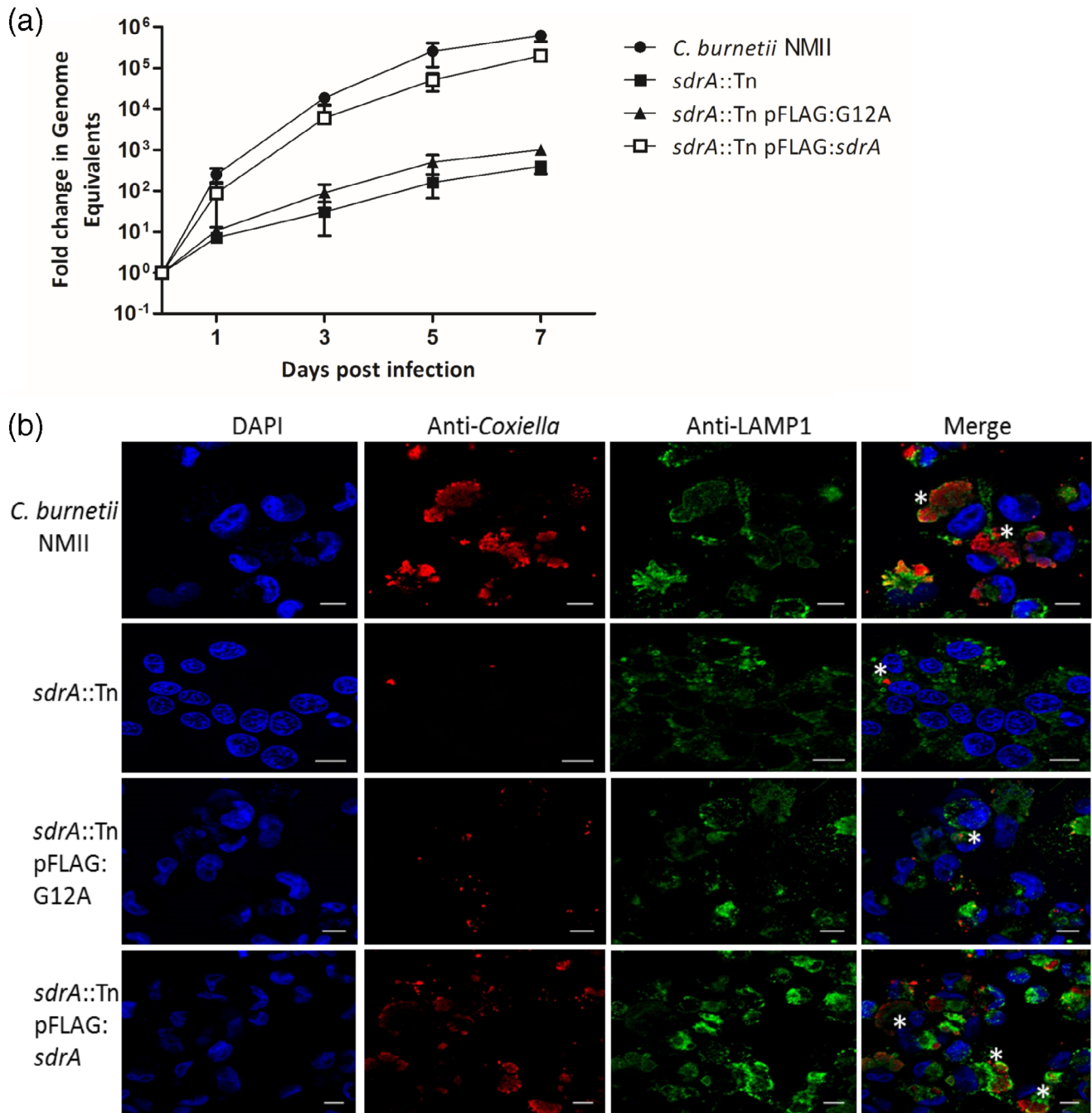


FIGURE 3 The intracellular replication defect of the *sdrA::Tn* mutant was not restored by complementation with a plasmid encoding the mutant G12A-SdrA protein lacking significant enzymatic activity. (a) Macrophage-like THP-1 cells were infected with *C. burnetii* strains for 7 days and the genome equivalents 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 $\pm$ 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 ($p < .05$ 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 μ m. White asterisks indicate representative CCVs. CCV, Coxiella-containing vacuole

similar, and pairwise comparison 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 ($p < .05$, Benjamini-Hochberg [BH]-adjusted t test) (Figure 6), varying by 3.9 to

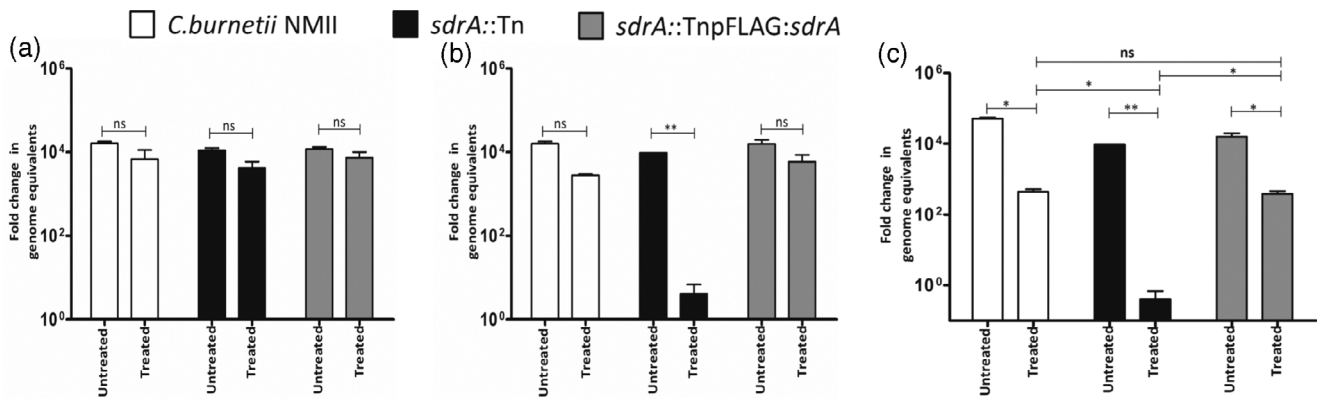


FIGURE 4 SdrA is required for resistance to oxidative stress induced by hydrogen peroxide treatment. *C. burnetii* replication is expressed as a fold change in genome equivalents after *C. burnetii* genomes were quantified by qPCR 7 days post-treatment with H₂O₂. All three strains displayed no growth defect after treatment with 50 μM H₂O₂ (a). The *sdrA::Tn* mutant is more susceptible to 500 μM H₂O₂ 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 1 mM H₂O₂ treatment (c), suggesting that these strains are also susceptible to higher concentrations of H₂O₂. 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 three times and significance determined by Student *t*-test (**p* < .05, ***p* < .01) relative to untreated or treated group. Error bars represent the standard deviation of mean (*n* = 3). ns = not significant (*p* > .05)

−4.02 log₂ fold change, whereas pairwise comparison of complemented mutant versus *sdrA* mutant revealed 29 metabolites to be significantly different in abundance (*p* < .05, BH-adjusted *t* test) (Figure 6), varying by a similar log₂ 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 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 PPP 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 [¹³C] 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 [¹³C] 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, these data demonstrate 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.

2.7 | Levels of 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

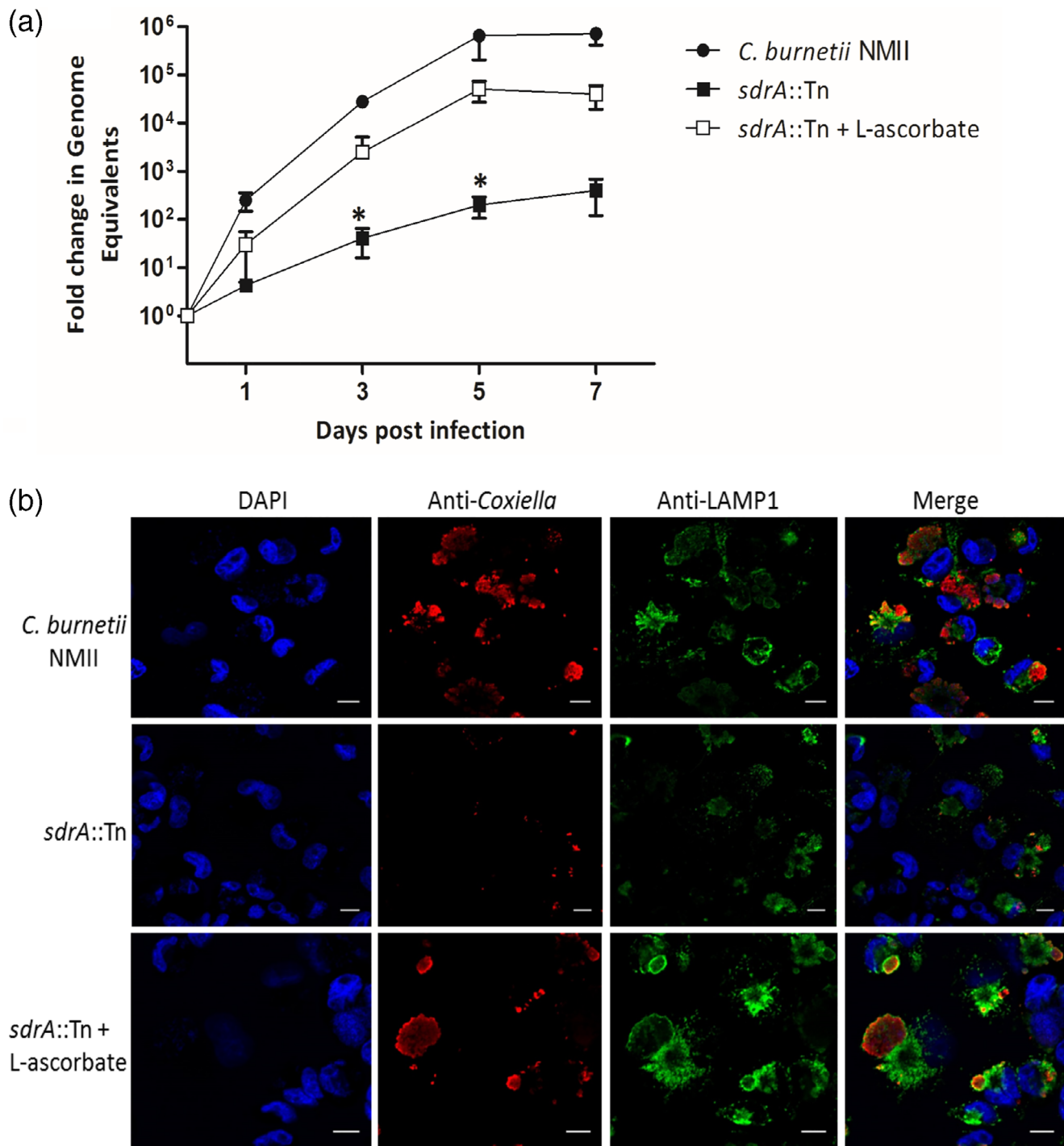


FIGURE 5 Supplementation of infection medium with 0.025 mM L-ascorbate resulted in a significant increase in replication of the *sdrA::Tn* mutant inside macrophage-like THP-1 cells (a). Intracellular replication of *C. burnetii* NMII (close circles), *sdrA::Tn* mutant supplemented with L-ascorbate (open squares) and *sdrA::Tn* mutant (closed squares) in THP-1 cells over a 7 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) ($p < .05$ 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 $\pm$ SD. (b) Immunofluorescent images showing THP-1 cells infected by *C. burnetii* strains for 3 days. The cells were fixed by 4% paraformaldehyde 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 μ m scale bar was used for these images that are representative of three independent infections. White asterisks indicate representative CCVs. CCV, *Coxiella*-containing vacuole

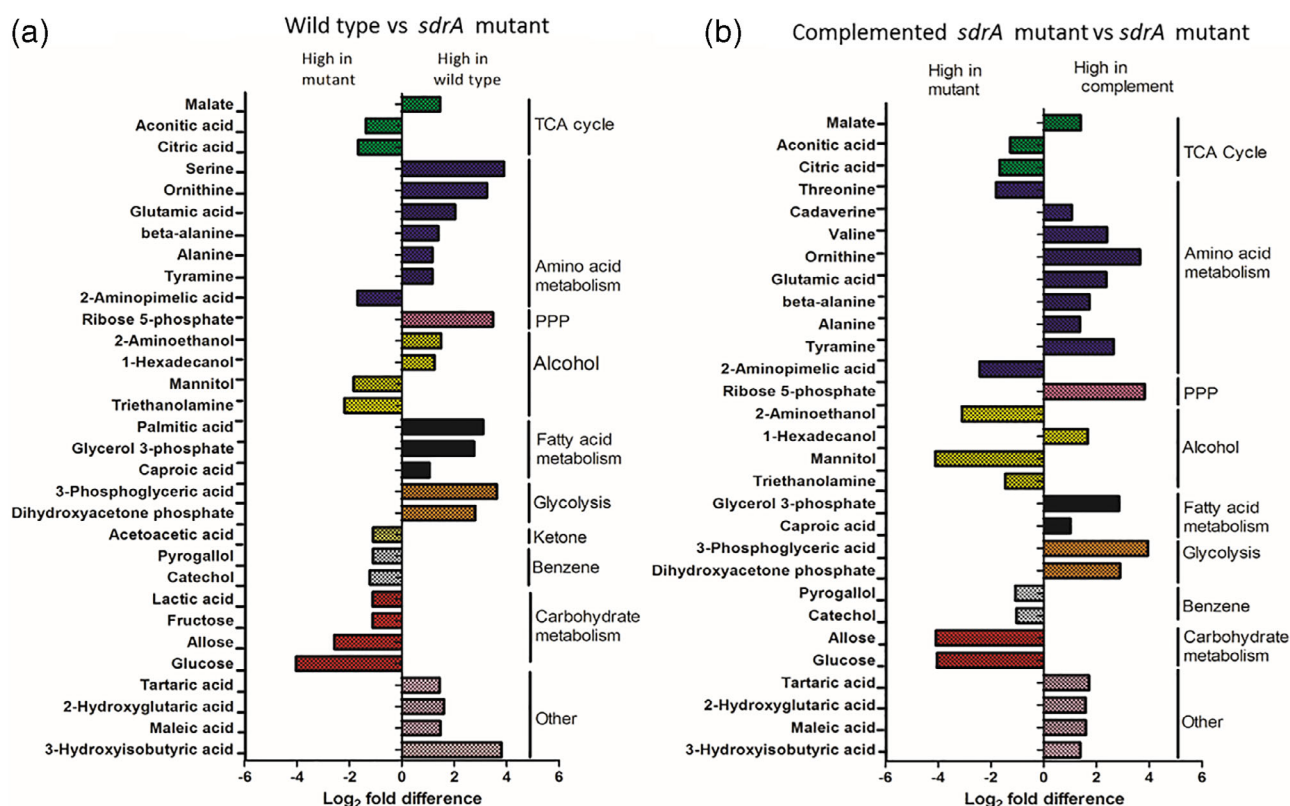


FIGURE 6 Metabolites that are significantly different ($p < .05$, Benjamini-Hochberg-adjusted t test) in abundance between *C. burnetii* NMII and *sdrA::Tn* (a) and between the complemented *sdrA* mutant and *sdrA::Tn* (b) in the GC/MS steady state analysis. The metabolites were identified from representative 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 comparison and are displayed as the average log₂ fold change of six biological replicates. GC/MS, gas chromatography-mass spectrometry

oxidative an environment the CCV actually is (Mertens & Samuel, 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.

3 | 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, Gennaris, Barras, & Collet, 2017). The defence mechanisms utilised by *C. burnetii* are only partly characterised and are predominantly inferred from genome annotation (Mertens & Samuel, 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 H₂O₂ to H₂O, with concurrent oxidation of Trx (Berggren et al., 2001; Ilnatko, Shaw, & Toman, 2012). Furthermore, *C. burnetii* expresses a DNA-binding peroxidoxin (CBU0963) that displays peroxidase activity that is also dependent on thioredoxin/thioredoxin reductase (Hicks, Raghavan, Battisti, & Minnick, 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 H₂O₂ as well as its greatly reduced intracellular replication. Methionine sulfoxide reductase (MsrA, CBU1306) is another enzyme involved in oxidative defence that requires reduced Trx (Ezraty et al., 2017; Mertens & Samuel, 2012), 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

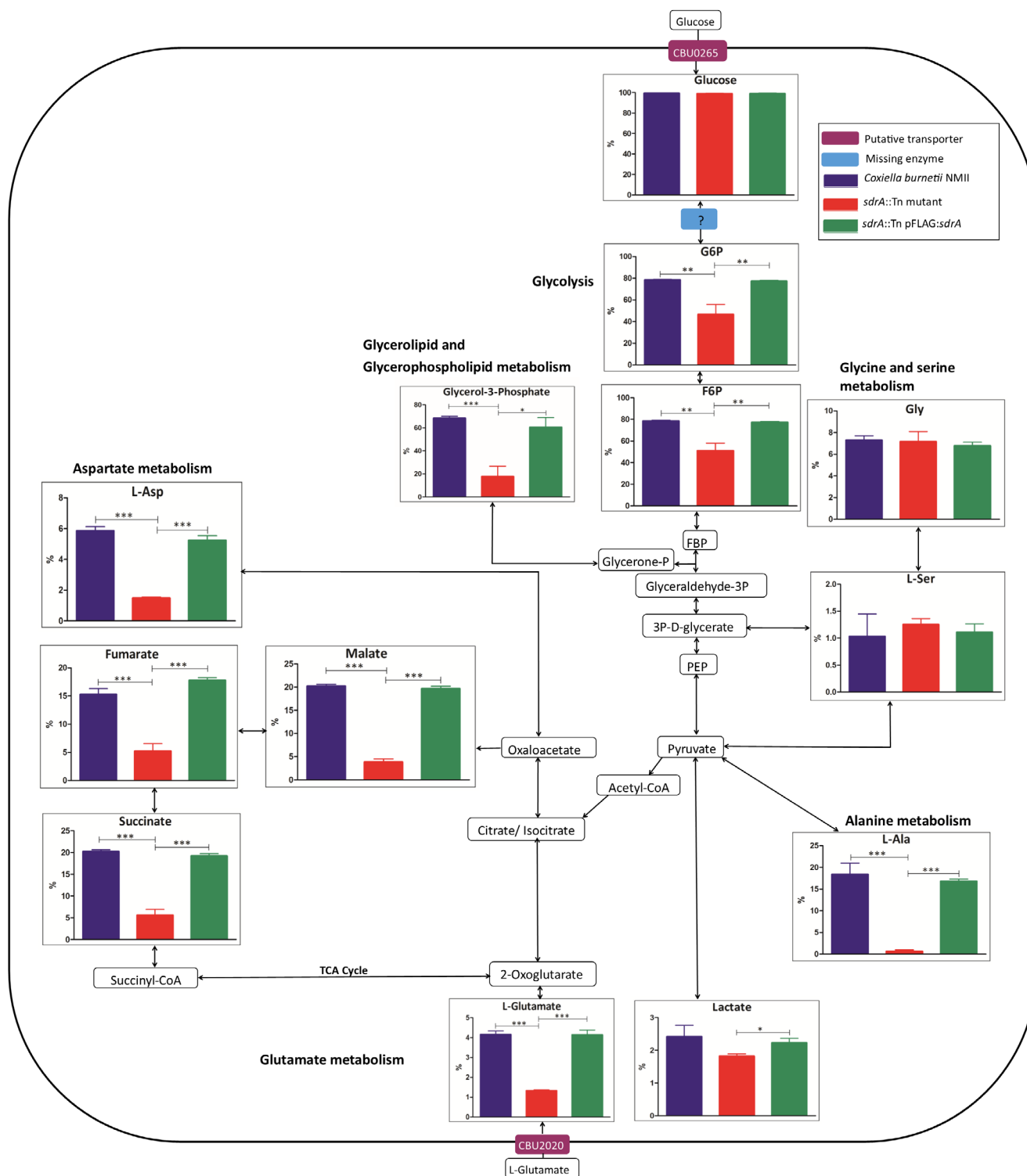


FIGURE 7 Labelling of intermediates of central carbon metabolism after one hour of labelling with ^{13}C glucose, as measured using GC/MS. The percentage of ^{13}C label incorporated into the total abundance of each metabolite is represented for *C. burnetii* NMII (blue), *sdrA::Tn* (red) and *sdrA::Tn pFLAG:sdrA* (green). Error bars represent the standard deviation of the mean from six biological replicates. * $p < .05$; ** $p < .01$; *** $p < .001$ (Student t test). 3P-D-glycerate, 3-Phospho-D-glycerate; F6P, fructose 6-phosphate; FBP, Fructose 1,6-bisphosphate; G6P, glucose 6-phosphate; GC/MS, gas chromatography–mass spectrometry; Gly, Glycine; Glyceraldehyde-3P, Glyceraldehyde 3-phosphate; Glycerone-P, Glycerone phosphate; L-Ala, L-Alanine; L-Asp, L-Aspartate; L-Ser, L-Serine; PEP, phosphoenolpyruvate; TCA, Tricarboxylic Acid Cycle

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

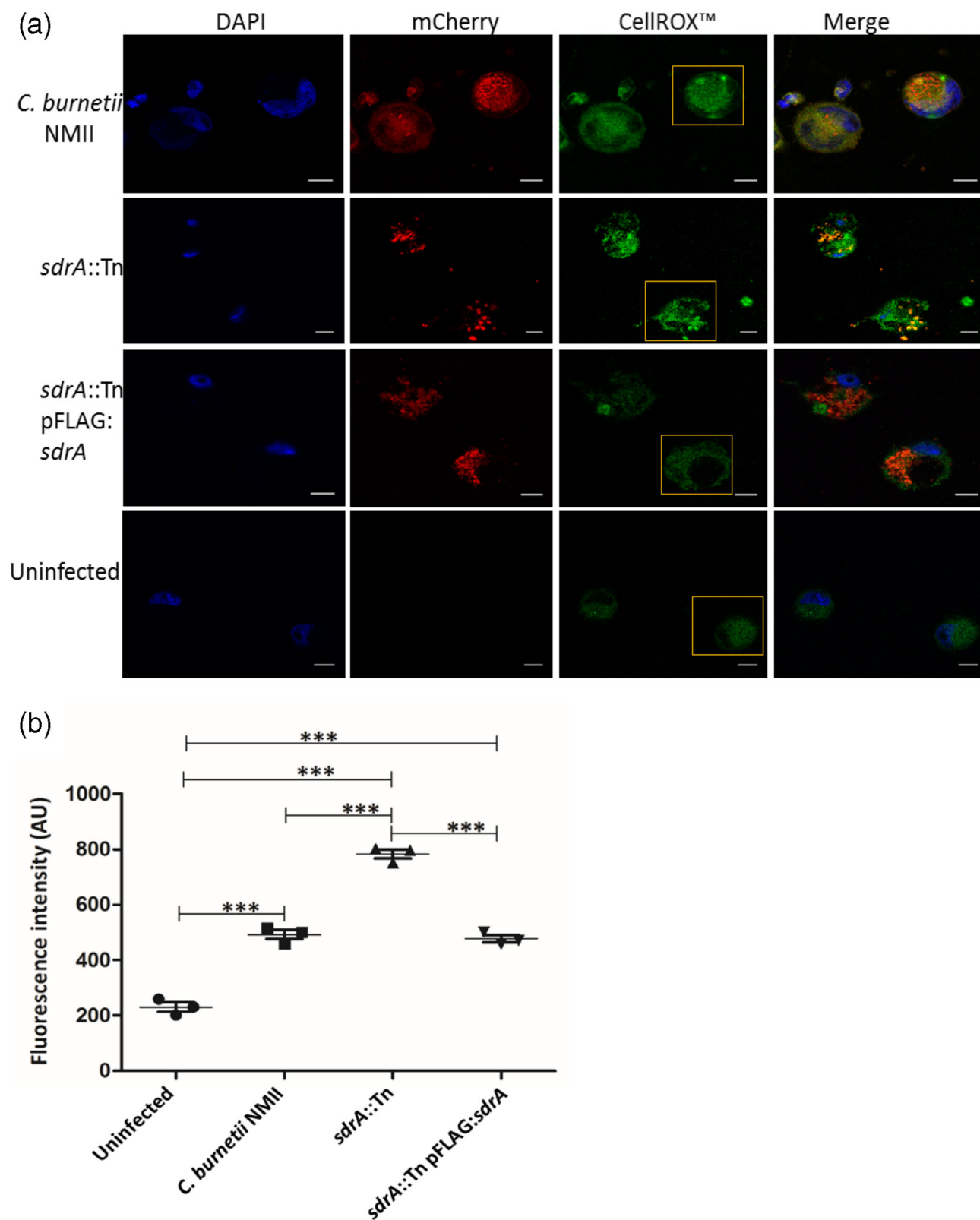


FIGURE 8 Legend on next page.

properly. The genome of *C. burnetii* encodes glutaredoxin (*grxC*; CBU1520) and the genes required for glutathione synthesis (Mertens & Samuel, 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 & Samuel, 2012). Overall, although *C. burnetii* appears to have a number of mechanisms to avoid and cope with oxidative stress (Mertens & Samuel, 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, Johnson, & Jarvis, 2001; Saffi, Sonogo, Varela, & Salvador, 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 & Heinzen, 2011; 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, To, Yamaguchi, Fukushima, & Hirai, 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 the 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, Mailloux, Puiseux-Dao, & Appanna, 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., Hackstadt & Williams, 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, Brandt, & Dräger, 2008; Kallberg, Oppermann, & Persson, 2010) and common substrates include alcohols such as ethyl (S)-(-)-4-chloro-3-hydroxybutyrate (Xue et al., 2011). Analysis of the

FIGURE 8 (a) Infection of THP-1 cells results in elevated host cell reactive oxygen species (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% paraformaldehyde for 15 min. Host cells were stained with DAPI (blue) and ROS 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 (three 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* < .001). CCV, *Coxiella*-containing vacuole

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 answer 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.

4 | EXPERIMENTAL PROCEDURES

4.1 | 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% CO₂ and 2.5% O₂ at 37°C. The selection of transposon mutants and plasmids were carried out using

chloramphenicol (3 µg/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% FCS at 37°C in 5% CO₂, respectively.

4.2 | 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 (3 µg/ml) as described previously (Omsland et al., 2011). 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*.

4.3 | Genetic complementation of the *sdrA* mutant

To genetically complement the *sdrA* mutant, full-length *sdrA* was amplified using specific primers containing engineered SalI restriction

TABLE 1 Primers used in this study

Name	Sequence (5' to 3')	Annealing temperature (°C)	Purpose
<i>sdrA</i> F1	AAAGTCGACATGCTTTTAAAGATAAAGTTGTTA	52	Complementation construct
<i>sdrA</i> R1	AAAGTCGACTCATTTAAAAATATCTAATGGAATT	52	Complementation construct
<i>sdrA</i> F2	CGCGGATCCATGCTTTTAAAGATAAAGTTGTTA	53	Cloning into pQE-30
<i>sdrA</i> R2	AAACTGCAGTCATTTAAAAATATCTAATGGAATT	53	Cloning into pQE-30
<i>ompA</i> F	CAGAGCCGGGAGTCAAGCT	55	Quantification of <i>Coxiella</i> genomes (Jaton, Peter, Raoult, Tissot, & Greub, 2013)
<i>ompA</i> 9 R	CTGAGTAGGAGATTGAATCGC	55	Quantification of <i>Coxiella</i> genomes (Jaton et al., 2013)
pKM225 F	TGCTCACATGTTCTTCTGTC	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	CGGATAACAATTTACACAG	48	Sequencing pQE-30-SdrA construct
pQE-30 R	GTTCTGAGGTCATTACTGG	48	Sequencing pQE-30-SdrA construct
G12A F	GTTATCGTAACAGCTTCCACGACAGG	55	Site-directed mutagenesis
G12A R	CCTGTCGTGGAAGCTGTTACGATAAC	55	Site-directed mutagenesis

Note: Bold represent Gly (GGT) mutated to Ala (GCT).

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 15000g at 4°C for 15 min 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 transferred 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% CO₂ and 2.5% O₂ at 37°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 & von Jagow, 1991) and transferred to the nitrocellulose membrane that was then probed with mouse anti-Flag IgG antibody (Sigma) diluted at 1/1,000 in 1% (w/v) skim milk in PBS-T and anti-mouse IgG HRP (GE Healthcare) diluted to 1/3,000 in 1% (w/v) skim milk in PBS-T before signal development using chemiluminescence.

4.4 | Intracellular replication assays in HeLa cells and THP-1 cells

HeLa CCL₂ cells were seeded into 24-well plates at a density of 5×10^4 with or without glass coverslips 1 day before infection. *C. burnetii* strains grown in axenic medium for 7 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 multiplicity of infection (MOI) of 50. Cells were infected by *C. burnetii* diluted in DMEM +5% FCS and incubated for 4 hr. The media were 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 H₂O 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 hr. Primary antibodies rabbit anti-*Coxiella* and mouse anti-LAMP1 were diluted in blocking buffer at 1/10,000 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 1/3,000. Before mounting on slides, DAPI diluted at 1/10,000 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 phorbol12-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.

4.5 | 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 the 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.

4.6 | 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 6xHis-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 hr by 1 mM isopropyl-beta-D-thiogalactopyranoside when the optical density of the culture reached 0.8. The cells were harvested by centrifugation at 7,700g 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) was 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 min. 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 NaH₂PO₄, 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.

4.7 | 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 min interval for 13 min using a spectrophotometer. Each enzyme assay was repeated three times.

4.8 | Induction of oxidative stress

C. burnetii strains were inoculated in ACCM-2 at 1×10^4 genome equivalents ml⁻¹ and treated with either 500 µM or 1 mM hydrogen peroxide (H₂O₂) before incubation for 6 days at 5% CO₂ and 2.5% O₂ 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.

4.9 | 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, 1,000g for 20 min following quenching of the cultures. The cells were briefly washed in ice-cold PBS and centrifuged again at 0°C, 1,000g for 20 min. The pellet was resuspended in 1 ml of ice-cold PBS and transferred to prechilled 1.5 ml microcentrifuge tubes for centrifugation at 0°C at 23,000g for 15 min and the pellet stored on ice until extraction. A 400 µl aliquot of extraction solution containing 3:1 (v/v) CH₃OH:H₂O with 1 nmol ¹³C₆-sorbitol and 10 nmol ¹³C₅, ¹⁵N-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 CHCl₃:CH₃OH:H₂O at the ratio of 1:3:1 (v/v/v) followed by vortexing and incubation for 10 min. The supernatant was transferred into a fresh microcentrifuge tube after centrifugation of the mixture at 0°C at 23,000g for 15 min. To induce phase separation, the supernatant was adjusted to CHCl₃:CH₃OH:H₂O ratio of 1:3:3 (v/v/v) by addition of dH₂O. 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 derivatised using methoxyamine (Sigma) and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS; Thermo Scientific) before analysis of polar metabolites using the Shimadzu GC-QQQ as described previously (Best et al., 2018; Masukagami et al., 2017).

4.10 | 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-normalised data were 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 BH adjustment to correct for false discoveries, with *p* < .05 considered significant.

4.11 | [¹³C] stable isotope labeling studies

[¹³C] glucose labeling was carried out by culturing *C. burnetii* as described for the steady-state analysis, followed by labeling for 1 hr in ACCM-2 supplemented with 11.11 mM [¹³C]-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 the DEXSI software (Dagley & McConville, 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.

4.12 | 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 min at 37°C. The medium was removed and the cells were washed three times with PBS. Cells were fixed with 4% paraformaldehyde for 15 min. Cells were washed with PBS before DAPI diluted at 1/10,000 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 for statistical analysis and graphing in GraphPad Prism. The data were 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.

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Fluorescent images were taken by confocal microscopes located at the Biological Optical Microscopy Platform, The University of Melbourne.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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Chapter 5. General discussion

5.1. Overview

The broad aim of this body of research was to investigate how the metabolism of *Coxiella burnetii*, a Gram-negative intracellular pathogenic bacterium that causes the zoonotic disease Q fever, is linked to its ability to replicate inside host cells and thereby cause disease. Identifying metabolic pathways required for intracellular replication can identify potential therapeutic targets, which will assist in the development of new drugs to treat and control the bacterium in both humans and animals. Although acute Q fever can be treated with currently available drugs such as doxycycline, the chronic infection requires several months of antibiotic treatment and is far more resistant to treatment (202). Doxycycline has been considered as a better option for treatment of chronic infections, but relapses were common due to the fact that *C. burnetii* replicates inside acidic phagolysosomes that have a pH of around 4.5 and this low pH in vacuolar compartments can significantly reduce the efficacy of drugs and inhibit the bactericidal activity (203). Furthermore, there are also some reports of resistance for doxycycline (203) and a more recent study showed that *C. burnetii* may overcome antibiotic-disturbed cell division by reducing the protein synthesis required for maintenance of cell envelope integrity and pH homeostasis (204). This study also showed that exposure to doxycycline activates defence against ROS in *C. burnetii* to prevent their deleterious effect. Therefore, this thesis research will help for developing new drugs targeting previously unexplored pathways that can shorten treatment time and avoid antibiotic resistance.

A Q fever vaccine (Q-VAX™, CSL, Australia) is currently licensed and available for humans in Australia (205). Q-VAX is the whole-cell formalin-inactivated vaccine of *C. burnetii* phase 1 organisms and considered acceptably safe for humans (205). There is also an animal vaccine (Coxevac™, Ceva Sante Animale, France), available in some European countries such as Netherlands and France, and which is not licensed in Australia (206). This vaccine is formaldehyde-inactivated *C. burnetii* phase 1 organism (206,207). Investigating *C. burnetii* attenuated mutants lacking metabolic pathways such as *de novo* NAD synthesis pathway might help to develop efficacious vaccine candidates. A previous studies has shown that an intracellular Gram-negative bacterium *Francisella novicida* transposon mutants attenuated for replication inside macrophages protected mice against wild-type challenge (208).

5.2. Metabolic pathways are required for intracellular replication

Although *C. burnetii* can now be cultured in axenic medium in the laboratory, it is considered an obligate intracellular pathogen in terms of its natural habitat. Like many obligate pathogens, *C. burnetii* lacks some metabolic pathways and relies on scavenging from the host. For example, *C. burnetii* is auxotrophic for 11 amino acids, such as arginine, cysteine, histidine, leucine, lysine, phenylalanine, proline, threonine, tyrosine, tryptophan, and valine (51). Most intracellular bacteria are dependent on exploitation of host amino acid metabolism (209,210). There is overlap between the amino acid auxotrophies of *C. burnetii* and other intracellular bacteria such as *L. pneumophila*, and the essential amino acids of the mammalian host cell (211,212). *L. pneumophila* is dependent on the host cell amino acid transporter solute carrier (SLC) protein SLC1A5 (213) and stimulates proteasomal degradation of polyubiquitinated proteins located at the vacuole (214). Endolysosomal compartments that fuse with *C. burnetii* CCV have several SLC proteins (215,216). Therefore, *C. burnetii*, similar to *Legionella*, may hijack peptides from host cell or amino acid transporter to get amino acids required for replication. Compared to some obligate intracellular bacteria like *Chlamydia* species, *C. burnetii* encodes more complete metabolic pathways, suggesting some of these pathways are essential for intracellular replication of the bacterium.

Obligate intracellular pathogens are dependent upon nutrients and metabolites from their host cells due to the extensive reduction of their genome size. This demonstrates that this metabolic dependency represents an Achilles' heel in the host-pathogen interaction and these obligate intracellular pathogens could be sensitive to changes in host cell nutrient and metabolite levels (217). The presence of redundant pathways in *Chlamydia* makes unclear on how precisely this pathogen acquire nutrients from the infected host cell, particularly amino acids. It acquires its amino acid requirements from the host cell through *de novo* biosynthesis, transport of environmental amino acids via amino acid transporters, and through degradation of polypeptides. Polypeptides endocytosed exogenously are delivered to the lysosome and are degraded by acid-activated proteases to oligopeptides and amino acids. The close proximity of chlamydial inclusions and lysosomes suggested that that *Chlamydia* obtains amino acids and/or oligopeptides from this organelle, which receives cargo from both endosomal and autophagosomal pathways (218).

Chlamydia do not possess any *de novo* NAD biosynthesis or salvage pathways and are dependent on direct uptake of NAD from its host (98) whereas *C. burnetii* possesses complete *de novo* NAD synthesis. This was further suggested by the previous transposon mutant library

screen (31) that identified a number of metabolic genes as required for intracellular replication. The overall aim of this thesis was to confirm the results of this screen for two genes, *nadB* and *sdrA*, and to investigate and characterise the molecular mechanism by which they are required for intracellular replication.

5.3. The importance of NAD and associated metabolites to *C. burnetii* pathogenesis

5.3.1. Synthesis and scavenging of NAD

NAD is an essential compound for all living organisms. The results of this research demonstrated firstly, that *de novo* NAD synthesis is necessary for *C. burnetii* intracellular replication, and secondly, that regeneration of NADP(H) by *C. burnetii* is also crucial. The necessity of *de novo* NAD synthesis was confirmed by the results in chapter 2, and is in contrast to other intracellular bacteria such as *Mtb. M. tuberculosis* is fully virulent in mouse models of infection in the absence of *de novo* NAD synthesis (84). This may be due to the fact that Pnc salvage pathways are more important for *M. tuberculosis* than *de novo* synthesis or can just compensate adequately for absence of synthetic pathway (84) as compared to *C. burnetii* which do not encode Pnc salvage pathways. The observed differences could also be due to the host cells. In this study human cell lines such as HeLa cells and THP-1 cells were used whereas the *M. tuberculosis* study was conducted in mouse models. Other prokaryotic cells such as *Haemophilus influenza* lacks *de novo* NAD synthesis and are dependent on salvage pathways (86) whereas the soil bacterium *Cytophaga hutchinsonii*, synthesise NAD via *de novo* pathway from Trp (87).

In the absence of *de novo* pathways, many prokaryotes scavenge NAD through salvage pathways or pyridine nucleotide cycling (95). Such pathways use major NAD precursors such as nicotinamide (Nm) and nicotinic acid (Na) which can be scavenged from the extracellular environment or breakdown products that are taken into the cell or produced intracellularly and fed into the NAD synthesis pathway, bypassing early *de novo* steps. For example, nicotinamide (Nm) is converted to nicotinic acid (Na) by the help of Nm deamidase (PncA) whereas another enzyme, Na phosphoribosyl transferase (PncB) facilitates the conversion of nicotinic acid (Na) to nicotinate mononucleotide (NaMN (95). The *Bordetella* species are dependent on uptake of Na or Nm because nearly all known species of this bacteria lack *nadA* and *nadB* genes and lacks *de novo* pathways that makes them to entirely rely on salvage pathways to generate NAD (95).

In addition, according to the previous study conducted by Prunier et al. (85), the pathogenic bacterium *Shigella flexneri* is a nicotinic acid auxotroph, unable to synthesize NAD via the *de novo* pathway, due to *nadA* and *nadB* gene mutations, and relies on the conversion of nicotinic acid to NaMN by PncB. When the functionality of *nadA/B* in *Shigella* was restored, a consistent loss of virulence and inability to invade host cells was observed suggesting that *nadA* and *nadB* are antivirulence loci. This observed differences between *Shigella flexneri* and *C. burnetii* suggests that targeting *nadB* may help to develop more specific and potent anti-microbials which can help combat the rising antibiotic resistance problem.

Other bacterial species such as *Streptococcus pyogenes* are also dependent on salvage pathway genes *pncA* and *pncB* for utilization of nicotinic acid (96). These Pnc salvage pathways are absent in *C. burnetii*, but the bacterium encodes an alternative salvage pathway via putative nicotinate phosphoribosyl transferase encoded by *cbu_1035* that may convert Na to NaMN (62). In chapter 2, there was no observable defect in replication of *nadB* mutants in axenic medium, suggesting this salvage pathway is active *in vitro*, and that the bacteria can uptake nicotinic acid from nutrient rich ACCM-2. However, this salvage pathway may not produce sufficient NAD for replication inside the cells in the absence of *de novo* synthesis or salvage pathways may not be active during intracellular replication.

Generally, the remarkable variations in NAD synthesis among the microorganisms shows that different organisms encode multiple salvage pathways from the same precursor (97). NAD metabolism has mainly evolved in various microorganisms towards auxotrophy or to a restriction in the capacity to use a biosynthetic precursor (73). Additionally, the diversity could be due to NAD-consuming enzymes such as sirtuins that are inhibited by nicotinamide (219) and may lead to a selection for evolution or acquisition of enzymes performing nicotinamide salvage (97).

5.3.2. Inhibition of *de novo* NAD synthesis

As most prokaryotes possess the *de novo* NAD synthesis pathway to produce NAD from L-aspartate, and animals synthesize NAD via a five step pathway from tryptophan (97) this prokaryotic-specific pathway is an ideal target for development of therapeutics.

In most prokaryotes, both NAD salvage and *de novo* synthetic pathways utilise the same downstream enzymes such as NadD and NadE. Both enzymes have been investigated as antimicrobial targets in *M. tuberculosis*, as inhibition of these enzymes has resulted in major metabolic catastrophe and bactericidal effect due to depletion of NAD(H) pool (83). However

the early steps of this pathway have not been investigated as drug targets, presumably as some bacteria, such as *M. tuberculosis*, do not require these enzymes for virulence (83). The results of chapter 2 suggest that investigating these earlier enzymes may identify antimicrobials with narrower specificities, which is also useful in combatting antimicrobial resistance.

The *de novo* NAD synthesis can be inhibited at the different stages of the pathway by various inhibitors which can completely block or reduce NAD synthesis in prokaryotes. In chapter 3, addition of 200 mM of meso-tartrate into the enzyme reaction inhibited the L-aspartate oxidase activity of NadB *in vitro*, reducing the activity of NadB to convert L-aspartate to iminoaspartate. This is in agreement with the previous study carried out in *E.coli* which showed that mesotartrate has completely inhibited L-aspartate oxidase activity of NadB. Meso-tartrate also inhibited axenic growth of *C. burnetii* although *nadB* mutants grow *in vitro* very well. This might be because meso-tartrate may have a broader effect on other metabolic pathways and biological processes of the bacterium. Previously, it was also reported that meso-tartrate has inhibited the growth of some salmonella strains *in vitro* which is assumed to be due to the inhibition of tricarboxylic acid (TCA) cycle enzymes (220), but the lack of toxicity for mammalian cells demonstrated in chapter 3 suggests this is not the case. This lack of cytotoxicity of meso-tartrate for mammalian cells also suggests this compound is a very good antimicrobial candidate for *C. burnetii*. The compound may also be a good therapeutic candidate for other intracellular pathogenic bacteria, such as *Legionella pneumophila*, *Salmonella* spp, *Mycobacterium tuberculosis*, *Ehrlichia*, *Brucella*, *Chlamydia* and *Listeria monocytogenes* and requires further investigation. This is because most of these vacuole forming intracellular bacteria share relatively similar features with *C. burnetii* such as replication inside macrophages and other phagocytic cells and they rely on host cells for nutrient acquisition (221). Furthermore, inhibition of synthesis of NAD⁺ and its derivatives may cause disruptions in redox metabolism and other central carbon metabolic pathways.

5.3.3. The importance of NADP(H) regeneration by SdrA

NADP(H) is a crucial cofactor involved in a number of biological reactions and, importantly, in anti-oxidative defence mechanisms due to its reducing power (222). Chapter 4 identified the importance of NADP(H) regeneration by the novel *C. burnetii* enzyme SdrA to intracellular replication and resistance to oxidative stress. This chapter answered the puzzle of NADP(H) regeneration by *C. burnetii* in the absence of the oxidative branch of the pentose phosphate pathway (PPP) (223). In addition to the oxidative PPP, NADP(H) can be synthesized via other

enzymes of central carbon metabolism such as isocitrate dehydrogenase (ICD) and malic enzyme (201).

A previous study carried out by isotope tracer assisted metabolite analysis using gas chromatography-mass spectrometry, and metabolic flux modelling showed that a cellulolytic bacterium *Clostridium thermocellum*, encodes inactive oxidative pentose phosphate pathway (224). However, the mechanisms how this pathogen synthesizes NADP(H) is still clearly unknown, but it may regenerate NADP(H) from isocitrate dehydrogenase as this enzyme is active in *Clostridium thermocellum* (224). However, the [¹³C] stable isotope labeling in chapter 4 showed significant changes in carbon flux in TCA cycle intermediates in the *sdrA* mutant. The significantly reduced incorporation of label into all TCA cycle intermediates in *sdrA* mutant suggested that ICD of *C. burnetii* is not regenerating sufficient NADP(H), and that the TCA cycle was in fact downregulated to reduce generation of the pro-oxidant NAD(H) to compensate for the inadequate supply of NADP(H) due to the loss of SdrA activity. The defence mechanisms used by *C. burnetii* against oxidative stress are not fully understood.

Yeast cells deficient in NADP(H) production showed a high sensitivity when exposed to oxidants (225). *Entamoeba histolytica* also do not encode oxidative branch of pentose phosphate pathway and when this parasite is exposed to oxidative stress, non-oxidative PPP metabolites has increased, a process attributable to an inhibition of glycolytic enzymes, resulting in redirection of the carbon flux (226). Although *Entamoeba histolytica*, do not possess oxidative branch of PPP, the non-oxidative pathway still responds in the presence of oxidative stress which suggests that the non-oxidative PPP plays an NADP(H)-independent role in the stress response in this parasite (225). The results presented in chapter 4 are the first report of defect in growth of an intracellular bacterium due to absence of NADP(H) regeneration, and suggest that further investigation of these metabolic pathways in intracellular pathogens may reveal more novel therapeutic targets.

5.4. Concluding remarks

The work presented in this thesis demonstrated the crucial role of both *nadB* and *sdrA* for *C. burnetii* replication and CCV formation. In this study human cell lines such as HeLa cells and THP-1 cells also served as a good model of infection to study *C. burnetii* pathogenesis using the phase II non-virulent strain. However, due to the lack of available physical containment level 3 facilities, animal experiments to investigate the role of NadB and SdrA *in vivo* were not able to be performed. Additionally, this lack of facilities limited investigation into the effect of

meso-tartrate on *C. burnetii* infected animals. Further investigation needs to be conducted to determine if *nadB* and *sdrA* attenuated mutants are useful vaccine candidates. Other future directions should also include investigation of the effect of meso-tartrate on other intracellular pathogens.

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