Copper homeostasis and *Salmonella* pathogenicity: Interplay with resistance to nitrosative stress

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in Molecular Microbiology in the Faculty of Life Sciences

2013

Thomas Richard Pointon

Table of contents

Total word count = 71,748

Index of Figures

Index of Tables

Abstract

Copper homeostasis and *Salmonella* pathogenicity: Interplay with resistance to nitrosative stress

Salmonella enterica serovar Typhimurium is responsible for a variety of diseases in domestic animals and humans. The infection of mice causes similar disease progression to human typhoid fever, thus representing a model for this systemic disease. The ability of S. Typhimurium to reside in a macrophage phagosome is important for their survival and spread to different organs. The antimicrobial mechanisms in this compartment include reactive oxygen species, reactive nitrogen species and elevated copper levels.

S. Typhimurium possesses two copper-exporting P_{1B}-type ATPases, CopA and GolT, both of which contribute to copper resistance. A previous study has shown that copper export by CopA and GolT confers a survival advantage in resting macrophage phagosomes. In this study the role of copper resistance systems has been examined further. The reduced survival of *ΔcopA/ΔgolT* in macrophages is detected beyond 8 hours post infection and coincides with increased nitrite production by macrophages. We have established that *ΔcopA*/*ΔgolT* display some increased sensitivity to reactive nitrogen species. However, whilst treatment of macrophages with the iNOS inhibitor L-NMMA reduced macrophage bactericidal activity against wildtype S. Typhimurium, this was not the case for *ΔcopA*/*ΔgolT*. In contrast, survival of *ΔcopA*/*ΔgolT* was not impaired in macrophages treated with the copper-chelator BCS. Furthermore real-time PCR confirmed the expression of *copA* and *golT* is elevated during infection of macrophages treated with IFN-γ or L-NMMA, but is reduced during infection of BCS treated macrophages. This indicates that bactericidal activity in macrophages is associated with copper availability and this is unaffected by reactive nitrogen species released due to iNOS activity.

In contrast to *Escherichia coli Salmonella* lacks a *cus* system associated with export across the outer membrane and hence the mechanism of copper export from the periplasm is not known. TolC was investigated as a potential outer membrane copper exporter based on clustering of TolC dependent systems to genes with sequence similarity to the S*. typhimurium* periplasmic copper chaperone CueP, across several bacteria. Mutation of *tolC* gave reduced copper tolerance and over-accumulation of copper at non-lethal concentrations under aerobic conditions. However TolC does not provide a role in copper tolerance or homeostasis under anaerobic conditions. TolC also does not provide tolerance or homeostasis to other divalent cations: Zn, Ni and Co. TolC therefore provides specific transport of copper under aerobic conditions in S. Typhimurium.

Declaration

No portion of the work referred to in the dissertation has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning

Copyright Statement

Acknowledgements

I would like to firstly thank my supervisor, Jen Cavet, for giving me the opportunity to undertake this study and for her amazing continuous support. Jen always made time to talk through issues or experiment ideas and gave me a great platform to work from. I would also like to thank my advisor, Ian Roberts, for giving critical analysis of work. As part of the Cavet lab group I had the opportunity to work with some fantastic scientists and I would like to thank them. I would like to especially thank Deenah Osman who gave me fantastic support and help to improve as a scientist, Kathryn Kirk who's enthusiastic assistance was help time after time and David Corbett who pushed me to interpret data to its full potential.

I would like to thank the members of Molecular Microbiology at the University of Manchester I had the pleasure to work with for their help and banter in particular: Ashley Houlden, Adrian Jervis, Helen Frost, Sean Weaver, Warren Flood, Sharaz Mohammed, Allison Wood, Eva Haas, Adrian Langarica, Julie Wang and Marie Goldrick. A thank you to the guys from Tuesday football for some great games and a nice mid-week break from the experiments.

I would like to thank University of Manchester staff who provided me with vital assistance through this study. Paul Lythgoe and the technicians within the Earth Sciences department for processing samples by ICP-MS analyses. David Brough and his lab group for supplying mice legs and teaching me how to extract bone marrow from them.

I would like to thank my boys: Anas Motlib, Bryan Chung, James Fernyhough and James Owen for giving me so much entertainment over the years and for always being there when I needed help. I would like to thank my mother, father and brother for providing a supportive loving environment to relax from the stress of work.

And finally the biggest thank you of all to my fiancèe Charlotte Collingwood for whom words cannot describe my gratitude for the: love, support and unfaltering confidence she has in me, which helped me overcome the challenging times during this study.

Without the help of the people I have thanked I could not have finished this PhD project and dedicate this work to them.

Abbreviations

Amp – Ampicillin

ATR - Acid tolerance response

ATP - Adenosine triphosphate

BCS - Bathocuproinedisulfonic acid

BSA - Bovine serum albumin

CAT – Chloramphenicol acetyltransferase

CCL - Chemokine ligand

Chl – Chloramphenicol

CFU – Colony forming units

cDNA – Complementary DNA

DNA – Deoxyribonucliec acid

DMEM – Dubecco's modified eagle medium

DMSO – Dimethyl sulphoxide

EDTA – Ethylenediaminetetraacetic acid

EGSC – *E. coli* Genetic Stock Centre

ELISA - Enzyme-linked immunosorbence assay

eNOS - Endothelial nitric oxide synthase

FAD - Flavin adenine dinucleotide

FBS – Foetal bovine serum

GDP - Guanosine diphosphate

GTP - Guanosine triphosphate

GSH - Glutathione

GSNO - S-nitrosoglutathione

GSSG - Glutathione disulfide

H_2O_2 - Hydrogen peroxide

HIV - Human immunodeficiency virus

HPA - Health Protection Agency

Hrs – Hours

ICP-MS – Inductively coupled plasma mass spectrometry

IFN-γ - Interferon gamma

IL- Interleukin

iNOS - Inducible nitric oxide synthase

IRF - Interferon regulatory factor

LB – Luria-Bertani

LPS - Lipopolysaccharide

MES – 2-(N-morpholino) ethanesulfonic acid)

MilliQ – Milli pore filtered water

Min – Minutes

MOI – Multiplicity of infection

N_2O - Nitrous oxide

N_2O_3 - Dinitrogen trioxide

NADPH - Nicotinamide adenine dinucleotide phosphate

NLR - Nod like receptor

nNOS - Neuronal nitric oxide synthase

NH_3 - Ammonia

NH_4^+ - Ammonium

$NO^{\cdot}$ - Nitric oxide

NO_2^- - Nitrite

NO_3^- - Nitrate

NOC 5 - 1-Hydroxy-2-oxo-3-(3-aminopropyl)-3-isopropyl-1-triazene

NOC 7 - 1-Hydroxy-2-oxo-3-(N-methyl-3-aminopropyl)-3-methyl-1-triazene

Nramp - Natural resistance associated macrophage protein

O_2^- - Superoxide

OD – Optical density

$OH^{\cdot}$ - Hydroxyl radical

$ONOO^{-}$ - Peroxynitrite

ONPG – Ortho – Nitrophenyl – β – galactoside

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

PFU – Plaque forming units

PSI – Pounds per square inch

qPCR – Quantitative PCR

RNA – Ribonuclear acid

RND - Resistance nodulation division

RNO - Nitrosated amide

RNS - Reactive nitrogen species

ROS - Reactive oxygen species

RSNO - Nitrosylated amide

RT- PCR – Reverse transcription PCR

SCV - *Salmonella* containing vacuole

SDS - Sodium dodecyl sulphate

Sec - Seconds

SGSC – *Salmonella* genetic stock collection

SPI - *Salmonella* pathogenicity island

SOD - Superoxide dismutase

T3SS - Type 3 secretion system

TBS – Tris buffered saline

TGF - Transforming growth factor

TLR - Toll like receptor

TNF - Tumour necrosis factor

Tris – Tris (hydroxymethyl) aminomethane

WHO - World Health Organisation

Chapter 1

Introduction

Salmonella remains a prevalent food borne pathogen that upon systemic infection takes residence within macrophages and macrophage like cells. Recent reports have identified copper as an antimicrobial agent within macrophages against intracellular S. Typhimurium (Osman *et al.* 2010). This study focuses on how copper exerts its antimicrobial effects in macrophages and the role of the S. Typhimurium copper resistance systems in defending against copper and other antimicrobial agents produced by macrophages during an immune response against *Salmonella*. Furthermore whilst previous work has provided detailed knowledge of *Salmonella* copper homeostatic mechanism within the cytosol and periplasm, it is largely unknown how copper is exported across the *Salmonella* outer membrane. This study investigates potential outer membrane copper exporters of *Salmonella*.

1.1 *Salmonella* the genus

The *Salmonella* genus is characterised by: Gram negative, rod-shaped, motile bacteria. *Salmonella* are a ubiquitous organism present in a variety of niches. The *Salmonella* genus is categorised into two separate species: *Salmonella enterica* and *Salmonella bongori*. *S. enterica and S. bongori* species account for more than 2500 serotypes. Serotypes are categorised into 6 sub-groups using the Kauffman-White scheme that divides serotypes based upon antigenic variation of; lipopolysaccharide (LPS, O antigen), capsule (Vi antigen) and flagella (H antigen, White 1926, Kauffman 1966). *S. enterica* subspecies I infect warm blooded hosts, whereas subspecies II-VII and *S. bongori* infect cold blooded animals. In rare circumstances warm blooded animals have been infected by *S. enterica* subspecies II-VII and *S. bongori* when immunocompromised (Aleksic *et al.* 1996, Giammanco *et al.* 2002).

1.2 *Salmonella* epidemiology and global impact

Food-borne pathogens remain a continual threat, with the World Health Organisation (WHO) estimating 1.8 million deaths being caused by food borne pathogens in 2005. The vast majority of these cases occurred within developing countries due to reduced hygiene in food preparation and a lack of medical infrastructure due to financial constraints. Over 9,000 *Salmonella* clinical cases were reported in England and Wales for 2010 (Health protection agency, HPA) and remain a present day food hygiene issue. Numerous *Salmonella*

outbreaks have been traced to a variety of food products including; mayonnaise, orange juice, bean sprouts and peanut butter (Jain *et al.* 2009, Ortega-Benito *et al.* 1992, Sheth *et al.* 2011, HPA). A common association of *Salmonella* mediated infections is from poultry, particularly eggs. *Salmonella* is capable of colonising the reproductive tract of hens and can be transmitted within eggs without any signs of contamination to the egg (Keller *et al.* 1995). However, live and attenuated *Salmonella* vaccinations are available for domestic animals but are not required by law. The introduction of vaccination within the egg laying industry directly correlates with a reduction of reported *Salmonella* infections. Prior to vaccination in 1993 >18,000 cases were reported which as previously stated has halved to approximately 9,000 cases during 2010 within England and Wales (HPA). *Salmonella* has also been detected within the meat of infected animals including cattle and chickens, water or produce exposed to faecal matter from infected animals (Abouzeed *et al.* 2000). Hence, there is a continuing risk of *Salmonella* infections from a variety of food sources as well as due to poor standards of hygiene.

1.3 *Salmonella enterica*

Food-borne pathogens cause infection through the faecal oral route, where infection occurs from the ingestion of contaminated food or water. Two *Salmonella* enterica serovars commonly associated with infection of humans are *Salmonella enterica subspecies serovar Typhi* and *Salmonella enterica subspecies serovar Typhimurium*. *S. typhi* is the aetiological agent for typhoid fever which is the systemic infection of *S. typhi* within a host. Usually S. Typhimurium cannot cross the gastrointestinal tract in humans and remains localised to the gut and leads to gastroenteritis. During infection of mice, however, S. Typhimurium is capable of crossing the gut epithelial barrier and causing systemic disease. As such, S. Typhimurium infection of mice is currently used as an infection model for studying systemic *S. typhi* infection in humans (McClelland *et al.* 2001). Although genetic differences are present between *S. typhi* and S. Typhimurium, the use of S. Typhimurium systemic infections as a model for *S. typhi* infection of humans provides important information (Felix *et al.* 1934, Parkhill *et al.* 2001).

1.3.1 *S.typhimurium* symptoms and disease

S. Typhimurium induced gastroenteritis symptoms include: diahorrea, fever, stomach pain and nausea (Hohmann 2001). Symptoms typically arise between 12-72 hours from initial

ingestion and last between 72 hours and two weeks. S. Typhimurium damages the gut epithelium inducing the release of fluids into the intestinal lumen giving diahorrea symptoms. S. Typhimurium can be shed within faeces for up to 10 weeks post infection (Santos *et al.* 2001). In cases of immunocompromised patients systemic S. Typhimurium infection has been observed but is a rare occurrence (Lepage *et al.* 1990). Mortalities caused by non-typhi *Salmonella* have increased in African countries due to increased numbers of the population suffering from human immunodeficiency virus (HIV) infection and consequently are immunocompromised (Sperber and Schleupner 1987).

1.3.2 *S. typhi* symptoms and disease

S. *typhi* infection of humans results in the systemic condition typhoid fever due to its ability to cross the gut epithelia and disseminate within the body, preferentially targeting the spleen and liver (Salcedo *et al.* 2001, Orskov and Moltke 1928). Symptoms of typhoid fever include fever, malaise and bloody stools (Santos *et al.* 2001). Under rare circumstances the gall bladder can become infected by *S. typhi* and periodically release *S. typhi* within bile secretions resulting in re-occurring infections and shedding within faeces until the gall bladder is removed (Gotuzzo *et al.* 1987). Typhoid fever has a mortality rate of approximately 15% without antibiotic treatment. Typhoid fever is treated with antibiotics but a rise in antibiotic resistance in the past 20 years has led to changes in the choice of antibiotics used. Resistance to fluoroquinolone, chloramphenicol, tetracycline and ampicillin has dramatically increased since early 1990s (WHO, Rowe *et al.* 1997). The rise of resistance to fluroquinolones has been directly attributed to the use of fluroquinolones within animal feed (Chiu *et al* 2002). Testing of meat from shop shelves identified the presence of antibiotic resistant strains of *Salmonella* supporting the hypothesis that drug resistant *Salmonella* are being passed on through the food chain (Threfall *et al.* 1996).

1.4 *Salmonella* pathogenesis

1.4.1 Surviving the stomach

Once *Salmonella* is ingested it proceeds to the stomach where it is exposed to a highly acidic pH as low as 1. The acidic pH provides an antimicrobial killing mechanism against harmful pathogens consumed. Mice deficient for gastric H^+/K^+ ATPase are unable to produce stomach acid and are more susceptible to food borne pathogens such as S. Typhimurium and *Campylobacter jejuni* (Tennant *et al.* 2008). *Salmonella* is capable of

surviving a highly acidic environment by the upregulation of an acid tolerance response (ATR). An ATR is first initiated at a mild pH of 5.5–6, in a pre-shock acid tolerance response. A secondary response is upregulated when *Salmonella* encounters a pH of <4 (Foster 1991). Without an initial pre-shock response *Salmonella* is more susceptible to killing at a low pH. Four regulators have been identified for providing a role in the upregulation of >40 proteins during an ATR which are: RpoS, OmpR, Fur and PhoP (Rychlik and Barrow 2005, Foster 1991). ATR proteins provide resistance to acidic pH by exporting H^+ from *Salmonella* or by consuming H^+ within a chemical reaction. A key ATR protein is the F_0F_1 H^+ ATPase that exports H^+ by an antiport mechanism internalising Na^+ or K^+, lowering cytosolic pH. Numerous food-borne pathogens possess a F_0F_1 exporter including: *Listeria monocytogenes*, *C. jejuni*, *Escherichia coli* and S. Typhimurium (Foster and Hall 1991, Birk *et al.* 2012, Conte *et al.* 2000). Numerous chemical reactions within *Salmonella* consume H^+ ions. Both lysine and arginine can be combined with H^+ to form an amine derivative lowering cytosolic pH. The addition of lysine and arginine to acid stressed *Salmonella* increased survival (Alvarez-Ordonez *et al.* 2010). *Salmonella* also increases the ratio of saturated: unsaturated fatty acids within its outer membrane which has been identified to increase resistance to acidic pH (Alvarez-Ordonez and Prieto 2010). An additional ATR responses include the synthesis of acid shock proteins that reduce the damage to macromolecules by altering cellular metabolism and molecular chaperoning (Audia *et al.* 2001).

1.4.2 *Salmonella* gut colonisation

Pathogenic bacteria that survive the acidic stomach conditions reach the gut and need to compete against an already established gut flora in a process termed colonisation resistance (van der Waaji *et al.* 1971). Colonisation resistance features of the gut flora include: competition for adhesion sites and production of toxins (Cursino *et al.* 2006). The ability of *Salmonella* to induce inflammation within the gut is a significant advantage for establishing itself within the gut flora. The commensal flora produces hydrogen sulphide (H_2S) which is toxic to the epithelial cells and is consequently converted to thiosulphate ($S_2O_3^{2-}$) by the caecum mucosal surface (Suarez *et al.* 1998). *Salmonella* cannot utilise thiosulphate as an electron acceptor but the immune response triggered by *Salmonella* results in the production of reactive oxygen species that oxidise thiosulphate to tetrathionate ($S_4O_6^{2-}$). *Salmonella* can use tetrathionate as a terminal electron acceptor for fermentation. Infections of mice with a *Salmonella* mutant unable to utilise tetrathionate as an electron acceptor gave an ~80 fold decrease in bacteria recovered from the gut compared to a parent strain (Winter *et al.* 2010). *Salmonella* can also respire phosphatidylethanolamine produced by the fermentation of

tetrathionate, giving a substantial advantage over the commensal gut flora (Thiennimitr *et al.* 2011). The ability to utilise tetrathionate and respire phosphatidylethanolamine gives no advantageous effects for systemic growth only colonisation of the gut. *Salmonella* can also out compete the gut flora by its ability to overcome zinc sequestration from the host zinc chelator, calprotectin, by the presence of a high affinity zinc importer system, ZnuABC (Lui *et al.* 2012).

Inflammation within the gut also promotes the expression of anti-microbial agents such as $Reg_{III}\beta$. $Reg_{III}\beta$ is a C-type lectin that binds to peptidoglycan damaging the external membrane of a bacterium. $Reg_{III}\beta$ provides antimicrobial activity against a number of gut commensal flora but S. Typhimurium is resistant to $Reg_{III}\beta$ killing. S. Typhimurium avoids $Reg_{III}\beta$ mediated killing due to its O-antigen reducing access to peptidoglycan although *E. coli* possess a similar O-antigen and are sensitive to $Reg_{III}\beta$ mediated killing (Stelter *et al.* 2011). Through avoiding killing by inflammation induced antimicrobials and reactive oxygen species broadening available nutrients, *Salmonella* is able to flourish during a gut immune response that does not benefit the commensal flora.

1.4.3 *Salmonella* crossing the gut epithelial barrier

Once *Salmonella* has reached the gut it begins to interact with the epithelial gut lining. Both S. Typhimurium and *S. typhi* interact with the gut epithelial barrier but S. Typhimurium cannot normally cross the human epithelial barrier. S. Typhimurium interacts with the gut epithelium and damages the epithelial barrier, in particular tight junctions between adjacent epithelial cells within 15 minutes of localisation (Jepson *et al.* 2000).

The recognition of antimicrobial agents of S. Typhimurium by the host induces an immune response through the expression of pro-inflammatory cytokines and will be discussed further in section 1.4.4. S. Typhimurium also utilises virulence factors that further augment the expression of pro-inflammatory cytokines (Tak and Firestein 2001). S. Typhimurium generates a stronger immune response within the gut than *S. typhi*. It is suggested that a reduced immune response may enhance the ability of *S. typhi* to cross the gut epithelium and cause systemic infection. *S. typhi* is capable of crossing the epithelial barrier by three separate mechanisms: by exploiting M cells (figure 1.1), after being phagocytosed by a $CD18^+$ cell which then itself crosses the gastrointestinal barrier; and can induce phagocytosis by non-phagocyctic cells (Jones *et al.* 1994, Vazquez-Torres *et al.* 1999).

Salmonella can cross the gut epithelial barrier through M cells typically within follicle-associated epithelium of Peyers patches. M cells provide a more open architecture for bacterial uptake than other cells within the intestine epithelia due to their role in sampling antigen content within the gut lumen. *Salmonella* induced damage to M cells provides an entry point to the gut associated lymphatic tissue (Jones *et al.* 1994). M cells present outside of the follicle-associated epithelium of Peyers patches have been identified to contain *Salmonella* after oral inoculation (Jones *et al.* 1994).

$CD18^{+}$ cells such as monocytes and macrophages as well as dendritic cells are able to uptake *Salmonella* from within the lumen of the intestine then subsequently cross the gut epithelial barrier and transport *Salmonella* within the blood stream (Vazquez-Torres *et al.* 1999). *Salmonella* is capable of diffusing the antimicrobial mechanisms that phagocytes employ against phagocytosed pathogens, this will be discussed in greater detail later (section 1.6). Survival within phagocytes enables *Salmonella* to utilise them as a method of transport without inducing an immune response (Vazquez-Torres *et al.* 1999).

Salmonella can initiate phagocytosis by a trigger mechanism where *Salmonella* induces its own uptake by the injection of virulence factors into a non-phagocytic cell by a type three secretion system (T3SS, section 1.4.4). The injected virulence factors cause the re-arrangement of the host cell cytoskeleton that uptakes *Salmonella* within a membrane bound vacuole. *Salmonella* has virulence factors encoded within regions of its genome termed *Salmonella* pathogenicity islands (SPIs) that are essential for virulence including T3SSs and effectors (Francis *et al.* 1993). These pathogenicity islands have been acquired during the evolution of *Salmonella* into a successful pathogen from its *Enterobacteriaceae* ancestor (Hacker and Carniel 2001). Several of the SPI encoded proteins are believed to be a result of phage transduction (Ehrbar and Hardt 2005, Ho *et al.* 2002).

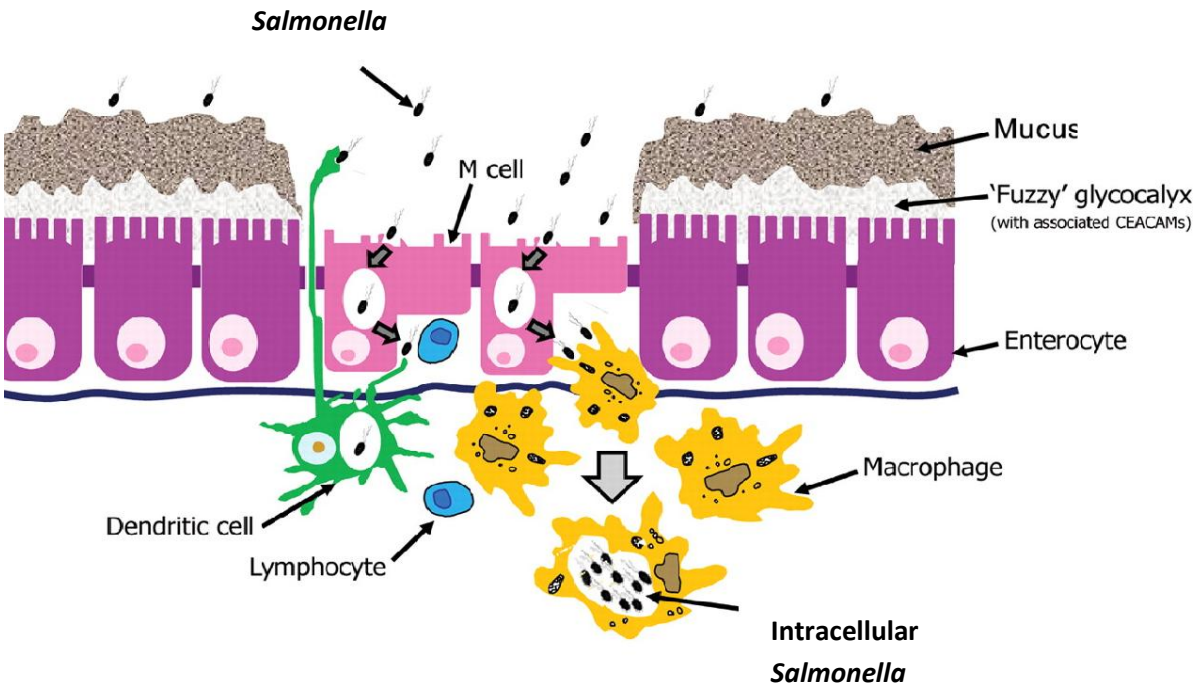


Figure 1.1 *Salmonella* can cross the gut epithelial barrier through M cells

Salmonella targets M cells within the gut epithelia to cross into the underlying lymphatic tissue containing multiple immune cells. *Salmonella* can cross the epithelia barrier by uptake within $CD18^{+}$ cells such as dendritic cells. *Salmonella* can also induce uptake in non-phagocytic cells such as enterocytes which do not have such an open architecture as M cells, due to M cells pinocytic nature and absence of mucus and glycocalyx layers (carbohydrate region of glycolipids and proteins within an enterocyte plasma membrane). Enterocytes are protected from *Salmonella* interaction with the plasma membrane by the presence of mucus and glycocalyx. *Salmonella* then disseminate around the body through the movement of macrophages leading to systemic infection. Adapted from Heijdan and Finlay (2012).

1.4.4 Triggering *Salmonella* uptake by a non-phagocytic cell

Upon reaching the intestine *Salmonella* pathogenicity island 1 (SPI1) is expressed. SPI1 is essential for inducing uptake within epithelial cells of the gastrointestinal tract. SPI1 encodes a T3SS; a hollow needle projection from the bacterium that injects virulence factors into the host cell cytosol that induce modification to its cytoskeleton arrangement (Lin *et al.* 2008). *Salmonella* mutants lacking a T3SS are avirulent (Watson *et al.* 1995, Galan 1999). SPI1 gene expression regulators include HilA, InvF and PhoPQ. HilA regulates the expression of

the T3SS and InvF regulates exported virulence factors, PhoPQ also induces expression of SPI1 virulence factors and will be discussed further in section 1.4.6 (Eichelberg and Galan 1999).

SPI1 effector proteins SipA and SipC perform important roles in modifying the host cell cytoskeletal structure. SipA interacts directly with actin filaments lowering the threshold concentration required for polymerisation and inhibits degradation of polymerised actin (Zhou *et al.* 1999). SipC also instigates actin polymerisation and cross-linking of actin filaments (Hayward and Koronakis 1999). The actions of SipA and SipC are additive despite no structural similarities between the virulence factors. SipA enhances SipC polyermerisation capabilities but only SipC is essential for uptake (McGhie *et al.* 2001). *Salmonella* also influences cytoskeletal structure by altering the host cell's signalling cascade. SopE and SopE2 are injected into the host cell and act as guanine nucleotide exchange factors that stimulate the release of guanosine diphosphate (GDP) giving increased efficiency of guanosine triphosphate (GTP) binding to Rho GTPases. SopE interacts with Rho GTPases Cdc42 and Rac whereas SopE2 only interacts with Cdc42 (Hardt *et al.* 1998). SopE enhances activity of Rho GTPase by almost 10^5 (Rudolph *et al.* 1999). SopB is an inositol phosphatase that induces actin cytoskeletal rearrangement through activation of Cdc42 through an independent pathway to SopE and SopE2 (Zhou *et al.* 2001). A *sopE/sopE2/sopB* triple mutant has severely inhibited cytoskeletal modification of host cells whereas single mutants exhibit no difference in host cell cytoskeletal modification to wildtype (Zhou *et al.* 2001). The combined actions of the SPI1 virulence factors mediate a change in membrane structure and triggers uptake of *Salmonella*.

Cell membrane ruffling is undone after *Salmonella* uptake by another SPI1 virulence factor, SptP. SptP acts as a guanine activating nucleotide protein increasing the speed of GTP hydrolysis and reducing enzyme activity, SptP reverses the cytoskeletal modifications after *Salmonella* is internalised (Fu and Galan 1999). SopE, SopE2 and SptP are injected into the host cell at the same time and induce opposing actions. SptP is degraded at a slower rate to SopE and SopE2. Once SopE and SopE2 have been degraded SptP can undo the modification to the cytoskeleton during uptake of *Salmonella* which is believed to be important in reducing detection of *Salmonella* by the immune system (Fu and Galan 1999).

Salmonella pathogenicity island 2 (SPI2) has been identified to be involved in the survival of the bacterium once internalised (Ochman *et al.* 1996). Although the distinct separation of the roles of each SPI is a simplistic view, further research has identified the requirement of SPI2 virulence factors for uptake and SPI1 virulence factors for intracellular survival (Coburn *et al.* 2005).

1.4.5 Systemic infection

Salmonella systemic infection whether *S. typhi* within humans or S. Typhimurium within mice is characterised by *Salmonella* residing within macrophages or macrophage like cells, typically those of the liver and the spleen. An interaction between *Salmonella* and a macrophage does not guarantee uptake of the bacterium, internalisation of *Salmonella* by phagocytosis occurs at a low rate of approximately 5% during *in vitro* infection experiments. A secondary infection of a macrophage already infected by a *Salmonella* bacterium occurs at an even lower percentage uptake rate (Gog *et al.* 2012). The internalisation of *Salmonella* modifies the elasticity of a cell structure implying that infected macrophages have modified structural properties (C. Bryant, University of Cambridge, private communication). *Salmonella* is uptaken within a vacuole termed the *Salmonella* containing vacuole (SCV) where it remains whilst within a macrophage. The number of *Salmonella* bacteria inside a host cell is variable with more than 50 bacteria being visualised although this is not a common feature with typically 1-3 bacteria being uptaken (Grant *et al.* 2009). When multiple *Salmonella* are internalised within a macrophage from independent phagocytosis events each bacterium has a separate SCV and when *Salmonella* replicates it is reported that the SCV also replicates (Eswarappa *et al.* 2010). The presence of multiple SCV's result in more targets for the host cell to attack spreading a macrophage's arsenal of antimicrobial defences, reducing the likelihood of killing all intracellular *Salmonella*. During an infection, the increase in internalised *Salmonella* is due to both replication and an increase of infected cells (Sheppard *et al.* 2003). *Salmonella* replicates within macrophages but the duration *Salmonella* remains within an infected cell remains variable. *Salmonella* can initiate host cell killing either within two hours of uptake by SPI1 inducing rapid killing by pyroptosis or after twelve hours by SPI2 mediated apoptosis (Monack *et al.* 1996). The rapid killing of the host cells seems counter-intuitive inhibiting *Salmonella* replication but results in a pro-inflammatory response and the recruitment of more macrophages and consequently have more targets to infect (Guiney 2005).

1.4.6 *Salmonella* adaption for intracellular survival

Phagocytosed pathogens follow a digestion pathway inside phagocytic cells resulting in the maturation of a phagosome. The maturation results in the acidification of the phagosome and fusion of lysosomes to destroy the internalised pathogen. *Salmonella* inhibits the fusion of lysosomes with the SCV by the actions of SPI2 encoded virulence factors (Buchmeier and Heffron 1991). SPI2 also encodes a T3SS (T3SS2) that injects effecter proteins into the host cell cytosol (Cid 2009). T33S2 is synthesised in response to nutrient limited conditions and acidic pH, both of which are environmental signals experienced within the SCV (Deiwick *et al*. 2002). SPI2 virulence genes are associated with survival inside host cells although SPI2 has been shown to be expressed prior to crossing the intestine (Brown *et al.* 2005). It has also been shown that mutations in SPI2 expression also affect expression of SPI1 genes (Deiwick *et al.* 1998). S. Typhimurium SPI2 mutants are avirulent in mice (Lee *et al.* 2000).

SpiC, an SPI2 virulence factor, inhibits the fusion of lysosomes with the SCV protecting *Salmonella* and inhibiting completion of the endocytic pathway (Uchiya *et al.* 1999). *Salmonella* reduces the number of lysosomes within a host cell as a possible contingency strategy that if lysosome fusion cannot be blocked the overall potency of lysosome digestive capabilities has been reduced (Eswarappa *et al.* 2010). *Salmonella* does not always successfully block lysosome fusion although lysosome fusion also does not always result in killing of *Salmonella* (Ishibashi *et al.* 1995).

SPI2 effectors enable the SCV to locate itself next to the Golgi apparatus where *Salmonella* encourages the transport of nutrients to the SCV (Salcedo and Holden 2003). SPI2 virulence factors also maintain the SCV integrity and prevent the maturation of the late endosome state of the SCV by the fusion of lysosomes. SifA induces the synthesis of *Salmonella* induced filaments around the SCV. SifA is essential for maintaining the SCV, a *sifA* mutant is released in the cytosol shortly after uptake where it is killed, and consequently *sifA* mutants are attenuated in mice (Stein *et al.* 1996, Beuzon *et al.* 2000). SPI1 also provides a role in maintaining the SCV as SPI1 mutants have a reduced phagosome size in comparison to wildtype *Salmonella* (van der Velden *et al.* 2000).

Salmonella within the SCV need to respond to changes within the macrophage to avoid killing by antimicrobial mechanisms. PhoPQ is a two component regulator that senses the external environment and alters gene expression accordingly. PhoQ is a cytoplasmic membrane sensor that detects varying levels of Mg^{2+}, Ca^{2+} or cationic peptides within the periplasm (Monsieurs *et al.* 2005, Murata *et al.* 2007). At micro molar levels of Mg^{2+}, a sign of an internalised environment, PhoQ self phosphorylates and transfers a phosphate group

to a PhoP (Groisman 2001), PhoP dimerises and binds to *pho* regulated promoters and modulates gene expression. Two component regulators are explained in greater detail later (section 1.9.2). Over 40 genes are regulated by PhoPQ; some of which provide key adaptations for intracellular survival such as the inclusion of aminoarabinose and fatty acids to the lipid A component of LPS that lowers the risk of detection by toll-like receptors (TLRs, Miller *et al.*1989, Soncini *et al.* 1996). PhoPQ also regulates the expression of both SPI1 and SPI2 inducing the expression of SPI1 genes in high Mg^{2+} levels as seen within the gut and inducing SPI2 genes in low Mg^{2+} levels such as inside a macrophage. PhoPQ can reduce the expression of SPI1 encoded virulence factors and flaggelin reducing the agents that TLRs can detect (Adams *et al.* 2001). PhoPQ upregulates SPI2 genes including low pH tolerance genes and genes involved in maintaining SCV integrity (Bearson *et al.* 1998). Mutants for *phoPQ* are avirulent highlighting the importance of continual adaptation by *Salmonella* during infection of macrophages (Monsieurs *et al.* 2005).

1.5 Host response to *Salmonella*

1.5.1 Host detection of *Salmonella*

S. Typhimurium infection of mice has revealed the importance of the recruitment of immune cells by inflammation in order to clear *Salmonella* infection. The recognition of *Salmonella* by the immune system is essential to mounting an immune response. Immune cells detect conserved pathogen traits called pathogen associated molecular patterns through receptors on their surface called pattern recognition receptors. A key family of pattern recognition receptors for detecting pathogens are the TLRs. TLRs induce expression of pro-inflammatory cytokines such as; interferon gamma (IFN-γ), tumour necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) (Kaiser *et al.* 2000). Each member of the TLR family bind specific bacterial ligands such as: TLR4 binds LPS, TLR5 binds flagellin and TLR9 binds unmethylated DNA (Lee *et al.* 2007). TLR4 is essential for providing an early inflammation response to *Salmonella* enabling macrophages to become activated (Talbot *et al.* 2009). TLR4 knockout mice have significantly reduced killing capabilities and are unable to remove a *Salmonella* infection (O'Brien *et al.* 1980, O'Brien *et al.* 1995). The recognition of flagellin by TLR5 is key for providing inflammation within the gut, stimulating the expression of IL-8 by nuclear factor kappa B (NF-κB) mediated expression (Yu *et al.* 2003). Flagellin mediated inflammation requires a functional SPI-1 and SPI-2 that provide inflammatory actions separately. SPI1 is required to induce flagellin mediated activation of caspase-1 (Miao *et al.*

2006). SPI2 is required for translocation of flagellin into the intestine epithelia upon contact (Lyons *et al.* 2004).

Another subset of eukaryotic immunoreceptors are the Nod like receptors (NLR), which also provide immunodetection of foreign agents. NLRs are located within the cytosol of a host cell and also bind PAMPs and stimulate an immune response upon activation. The NLR family also bind varying substrates depending on the receptor, as previously stated with the TLR family. NLR3 responds to pore-forming toxins or extracellular adenosine triphosphate (ATP); whereas NLR4 binds a different region of flagellin to that of TLR5 and is essential for upregulating caspase-1 (Koo *et al.* 2008, Franchi *et al.* 2007). Upon binding the correct antimicrobial molecule a NLR oligomerises binding a recruitment protein that contains a caspase-1 recruitment site, caspase-1 is recruited and activated forming an inflammasome that can activate IL-1β and inactive IL-18 and initiate cell death by pyroptosis (Lara-Tejero *et al.* 2006).

1.5.2 Immune response against *Salmonella* infection in the gut

The immune system responds to the infection of *Salmonella* within the gut and *Salmonella* potentiates the immune response through the actions of its injected virulence factors. SipC binds and activates caspase-1 converting inactive IL-1β and IL-18 into their active forms producing a pro-inflammatory immune response (Hersh *et al.* 1999). The activation of caspase-1 can initiate pyroptosis of the host cell; this is a form of cell death that is distinguishable from apoptosis (Hobbie *et al.* 1997). Pyroptosis is caspase-1 dependent degradation of DNA but the nucleus retains its integrity during degradation unlike apoptosis when nuclear fragmentation is visible (Bergsbaken *et al.* 2009). SopB, SopD, SopE, SopE2 and SipA modification of the host cell cytoskeleton activates mitogen associated protein kinases particularly Cdc42, that results in the downstream signalling activation of the transcription factor NF-κB. NF-κB upregulates the expression of pro-inflammatory chemokines; IL-1, IL-6, IL-8 and TNF-α (Tak and Firestein 2001).

1.5.3 Immune response against systemic *Salmonella* infection

The initial immune response to *Salmonella* consists of the innate immune system, specifically: dendritic cells, macrophages and neutrophils. The innate immune system aims to both kill *Salmonella* and present *Salmonella* antigens to T and B cells activating the adaptive immune system (Rosenberger and Finlay 2003, Kalupahana *et al.* 2005).

Salmonella has developed several mechanisms to avoid killing and inhibit antigen processing. Once macrophages encounter *Salmonella* they phagocytose *Salmonella* and produce various antimicrobial stresses including: reactive oxygen species (ROS), reactive nitrogen species (RNS) and varying metal ion levels. It is currently not known which receptors are involved in the recognition and uptake of *Salmonella* by phagocytosis. *Salmonella* preferentially resides within macrophages due to their ability to detoxify the internal antimicrobial threats and avoid further immune responses such as complement.

Neutrophils also uptake *Salmonella* and have increased killing efficiency against *Salmonella* than macrophages. Reports have confirmed *Salmonella* is killed and does not replicate within neutrophils unlike macrophages (Weiss *et al.* 1982). Macrophages can kill *Salmonella* but require IFN-γ or LPS activation prior to infection to do so (Gilberthorpe *et al.* 2007).

Dendritic cells uptake *Salmonella* within the gut. *Salmonella* is able to inhibit its degradation and antigen presentation unless opsonised (Tobar *et al.* 2006). Virulence factors within SPI2 such as SifA and SopD2 inhibit antigen presentation which prevents the activation of immature T cells within lymph nodes and an adaptive immune response (Cheminay *et al.* 2005). *Salmonella* can still be killed by the adaptive immune system by virtue of cytotoxic and natural killer T cells. The innate immune system also upregulates an immune response through the release of cytokines and chemokines. Pro-inflammatory cytokines and chemokines: IL-1β, IL-18, chemokine ligand 2 (CCL2) and CCL20 stimulate the recruitment of more leukocytes to the gut (Burkey *et al.* 2007).

The adaptive immune system gives a polarised response based on the location of the pathogen. The available options for an adaptive immune response are an intracellular infection response, T helper-1 or an extracellular immune response, T helper-2. *Salmonella* induces a Th1 response due to its preference to reside within macrophages. Key cytokines in a Th-1 response are: IL-12, IL-18 and IFN-γ. IL-12 and IL-18 are produced by macrophages that have encountered *Salmonella* and stimulate the expression of IFN-γ by T cells, natural killer cells, neutrophils and macrophages (Ramarathinam *et al.* 1991, Kirby *et al.* 2002). IFN-γ activates macrophages to produce significantly greater antimicrobial threats against *Salmonella*, which as the main niche for *Salmonella* survival is key to their killing. IFN-γ activation stimulates increased expression of iNOS that produces nitric oxide; nitric oxide contributes to increased killing of intracellular *Salmonella* and is further discussed in section 1.6.3. Mice knockouts for IFN-γ have significantly reduced survival upon infection with S. Typhimurium. The administration of IFN-γ into an IFN-γ deficient mice give a significant reduction in the number of S. Typhimurium within organs (Nauciel and Espinasse-Maes 1992, Matsumura *et al.* 1990). The exact antimicrobial mechanisms by which

Salmonella is killed remains unknown and is believed to be from the collaborative effort of several antimicrobial mechanisms working in combination.

1.5.4 Natural resistance associated macrophage protein (Nramp)

Nramp is a family of transporters that transport divalent metal ions. The eukaryotic Nramp1 transporter associates with a phagosome and has been shown to provide resistance in macrophages against intracellular pathogens such as *S. typhi*, *M. tuberculosis* and *Leishmania donovani* (Crocker *et al.* 1984, Plant *et al.* 1982). Nramp1 transports divalent cations across the phagosomal membrane by a H^+ antiport mechanism, although the direction of transportation and the metal transported remains an area of debate. One hypothesis is that iron is transported into the phagosome and catalyses the formation of reactive oxygen species by Fenton chemistry (Goswami *et al.* 2001). Research groups Zwilling *et al.* (1999) and Kuhn *et al.* (1999) have identified increased import of iron into phagosomes and increased levels of hydroxyl radical formation compared to an *nramp1* mutant. Although an alternate theory is that iron is transported out of the phagosome by Nramp1 limiting iron levels that are required for bacterial growth (Gomes and Appelberg 2002). Phagosomes from macrophages with functional Nramp1 exclude manganese divalent ions to a greater capacity than *nramp1* mutant macrophages supporting the hypothesis that Nramp1 is involved in the removal of Mn^{2+}, an important trace metal (Kehres *et al.* 2002). Several polymorphisms of *nramp1* have been identified, humans have varying resistance or susceptibility to intracellular pathogens dependent on the polymorphism they encode (Blackwell and Searle 1999).

1.6 Reactive species

Internalised pathogens are exposed to several antimicrobial threats. Reactive species are produced by phagocytes to kill and inhibit replication of phagocytosed pathogens.

1.6.1 Reactive oxygen species

Reactive oxygen species are oxygen based ions with an unpaired electron. Superoxide (O_2^-) is synthesised by the multi-subunit enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which is assembled at the site of phagocytosis and incorporated into the phagosome membrane (Aljada *et al.* 2002). NADPH oxidase consists of five Phox subunits:

40, 47, 67, 22 and 91. A mutation in any of the *phox* genes that encode the subunits: 22, 47, 67 and 91 results in a condition termed chronic granulomatos disease. Humans suffering from chronic granulomatos are subject to re-occurring infections by catalase positive pathogens such as S. Typhimurium, *Staphylococcus aureus* and *Aspergilus fumigatus* (Assari 2006). Studies have identified that the administration of IFN-γ improves patient resistance to catalase positive pathogen infections (Malmvall and Follin 1993).

Oxygen is reduced to superoxide in a two step process; NADPH electron buffering system provides an electron to flavin adenine nucleotide (FAD), FAD then transfers the electron to the haem group of NADPH oxidase that reduces oxygen (Koshkin *et al.* 1993). The electron configuration of superoxide and other reactive species are shown within figure 1.2. Superoxide can readily interact with a pathogen or can react with other antimicrobial molecules such as: reactive nitrogen species or chlorine to provide varying antimicrobial agents (Bannister and Bannister 1985). Reactive oxygen species do not combine with chlorine within macrophages but do so in neutrophils through the action of the enzyme myeloperoxidase that produces hypochlorous acid (Klebanoff 1967). The presence of myeloperoxidase within neutrophils is believed to contribute to neutrophils having greater ability to kill *Salmonella* than macrophages. Superoxide and nitric oxide ($NO^{\cdot}$) can combine to form the reactive species peroxynitrite ($ONOO^{-}$). Peroxynitrite is a highly oxidising compound capable of damaging multiple macromolecules and will be discussed in greater detail later (section 1.6.5).

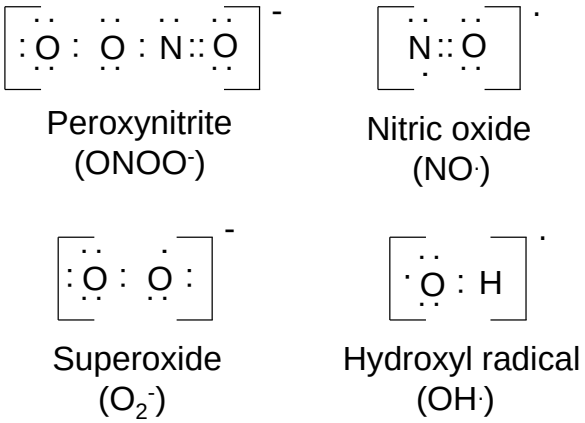


Figure 1.2 Electron configurations of peroxynitrite, nitric oxide, superoxide and hydroxyl radical

Outermost electron shell of reactive species displayed with dots representing electrons. Peroxynitrite and superoxide contain one extra electron than the oxygen and nitrogen atoms that constitute the molecules would typically contain, giving the molecule a negative charge. Nitric oxide and hydroxyl radical both have an unpaired electron and require one electron to obtain a full outer-shell, consequently both are highly reactive in an attempt to gain an electron.

The negative charge of superoxide inhibits passive diffusion across a membrane and consequently enters *Salmonella* through porins in the outer membrane (Imlay and Imlay 1996). It is uncertain how superoxide crosses the cytoplasmic membrane. The oxidation of DNA and increase in reactive oxygen stress response genes indicate that superoxide and other reactive oxygen species cross the cytoplasmic membrane in Gram negative organisms (Sakai *et al.* 2006). Another reactive oxygen species compound encountered during internalisation of a macrophage is hydrogen peroxide (H_2O_2). By definition hydrogen peroxide is not a reactive oxygen species but an intermediate molecule that is capable of producing highly potent hydroxyl radicals ($OH^{\cdot}$). Hydroxyl radicals are formed when hydrogen peroxide is reduced in the presence of a redox agent such as a metal by undergoing Fenton chemistry (Fenton 1894). Due to the uncharged nature of hydrogen peroxide it is capable of passively diffusing across a membrane. The risk of Fenton chemistry occurring between freely diffusible hydrogen peroxide and metals within the cytosol is a possible factor of why metal homeostasis is tightly regulated within several pathogenic organisms. Sources of hydrogen peroxide include production by *Salmonella* itself during aerobic respiration from the leakage of electrons within the electron transport chain reducing water in the presence of a hydrogen ion. The production of hydrogen peroxide during respiration is estimated to be between 1-4% of oxygen consumption (Gonzalez-

Flecha and Demple 1995). Hydrogen peroxide is also produced from the dismutation of superoxide by superoxide dismutase enzymes.

Reactive oxygen species damage and kill intracellular pathogens through targeting: DNA, proteins and lipids (Fang 2004). Reactive oxygen species can oxidise nucleotide bases and proteins containing tyrosine, cysteine and methionine residues altering their structure (Wefers *et al.* 1987). Reactive oxygen species damage lipids by lipidperoxidation of unsaturated lipids particularly within bacterial membranes, the reactive nitrogen specie nitrite ion ($^{\cdot}NO_2$) can also initiate lipid peroxidation. The peroxidation of unsaturated lipids can induce a chain reaction of lipid peroxidation shown in figure 1.3 (Girotti 1985). Lipidperoxidation can also transfer electrons to other ligands spreading the damaging effects to other locations within a bacterium.

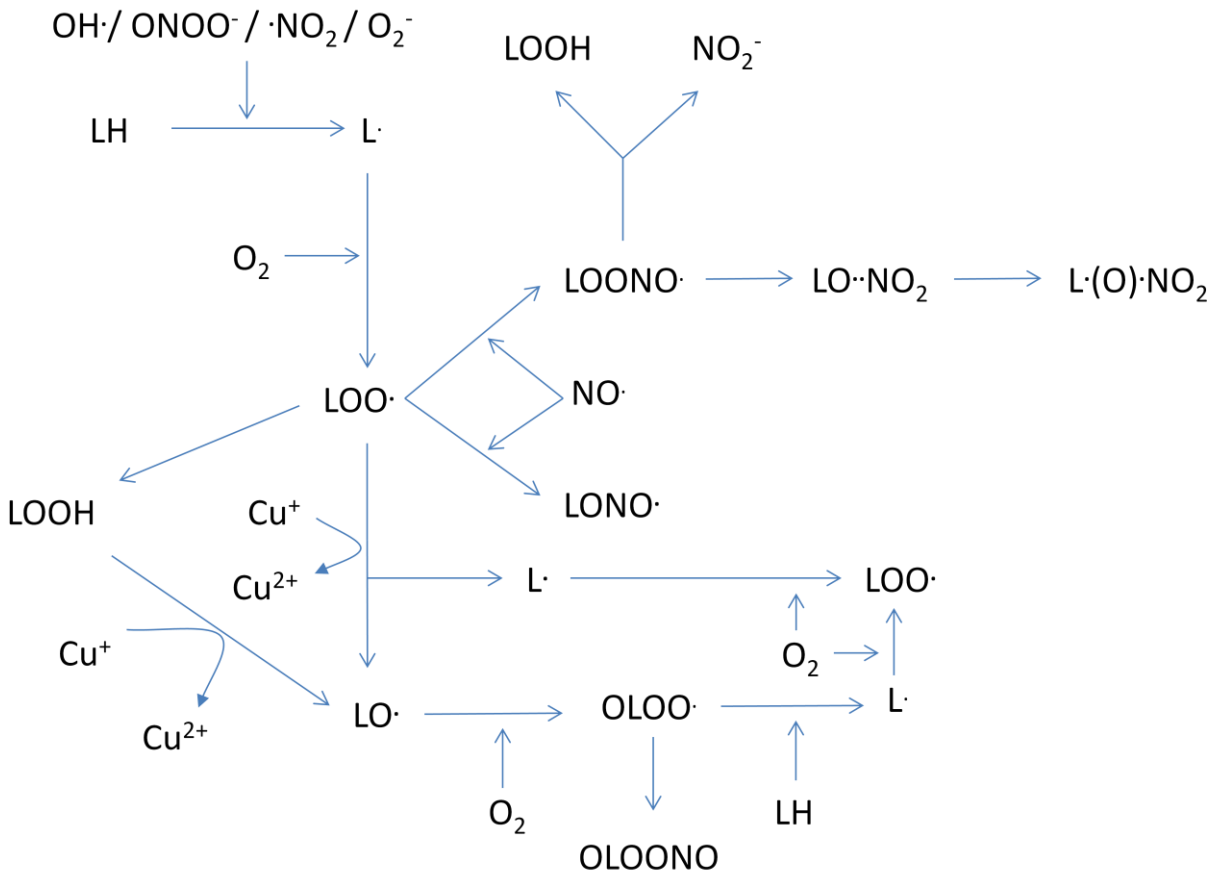


Figure 1.3 Reactive oxygen species generation of lipid peroxidation

Several nucleophiles; $OH^{\cdot}$, $ONOO^{-}$, $^{\cdot}NO_2$ and O_2^{-} can initiate the formation of a lipid radical by attacking the covalent carbon-carbon double bond in a mono or poly-unsaturated lipid. The oxidation of a lipid radical to give lipid peroxide can then act as a nucleophile to another unsaturated lipid giving rise to another lipid radical whilst remaining a lipid radical itself. Copper can also play a significant role in potentiating lipid peroxidation.

1.6.2 *Salmonella* detoxification of reactive oxygen species

Salmonella directly prevents reactive oxygen species damage by inhibiting the localisation of NADPH-oxidase containing vesicles to the SCV by SPI2 virulence factors (Vazquez-Torres *et al.* 2000). In the event *Salmonella* is exposed to reactive oxygen species several reactive oxygen species detoxification pathways are upregulated. Three transcription regulators: OxyR, SoxR and RpoS have been identified to provide a response to reactive oxygen species within *Salmonella*. OxyR is the most important reactive oxygen species response transcription factor and upregulates an oxidative stress response in both *E. coli* and S. Typhimurium (Blanco *et al.* 1995). OxyR contains two conserved sulphur amides at positions 199 and 208 that under oxidative stress form a disulphide bond that enables OxyR to dimerise and upregulate a change in gene expression. OxyR upregulates multiple reactive oxygen species detoxification systems including; nonspecific binding protein (*dps*), glutathione reductase (*gor*), glutaredoxin (*grxA*), catalase (*katG*) and akyl hydroperoxide reductase (*aphCF*) (Christman *et al.* 1985, Morgan *et al.* 1986). SoxR senses reactive oxygen species through a [2Fe-2S] cluster, that in the presence of reactive oxygen species becomes oxidised, and upregulates expression of SoxS (Krapp *et al.* 2011). The mechanism by which SoxR is activated by reactive oxygen species is currently unclear and is hypothesised to be dependent upon the oxidation state of the NADPH/$NADP^+$ cytosol pool. SoxS enhances the upregulation of the SodA Mn-containing superoxide dismutase providing detoxification of superoxide (Fang e*t al*. 1997). RpoS upregulates the expression of catalases KatE and KatN and DNA exonuclease III to remove oxidised nucleotides (Eisenstark *et al.* 1996).

Glutathione (GSH) can also scavenge reactive oxygen species and promote their degradation, glutathione peroxidase (ButE) degrades hydrogen peroxide in a glutathione dependent manner, and glutathione is discussed further in section 1.7.2 (Chesney *et al.* 1996, Arenas *et al.* 2010). *Salmonella* also has an abundance of enzymes to detoxify superoxide and hydrogen peroxide. Superoxide dismutases (Sod) detoxify superoxide into hydrogen peroxide as show below.

$O_2^- + 2H_2O \rightarrow 2H_2O_2$

Salmonella contains four Sod enzymes; two Cu, Zn Sods, ($SodC_I$ and $SodC_{II}$) in the periplasm and two cytoplasmic Fe (SodB) and Mn (SodA) Sod enzymes. All four superoxide dismutase enzymes detoxify superoxide, the redundancy emphasises the importance of superoxide detoxification to *Salmonella*. A double mutant for *sodA/sodB* is highly susceptible to reactive oxygen species, exhibiting killing at μM concentrations in comparison to wildtype

that is capable of survival mM concentrations (Craig and Slauch 2009). The periplasmic Cu, Zn Sod enzymes do not provide as essential role in superoxide detoxification as observed for the cytoplasmic Sod enzymes. $SodC_I$ and $SodC_{II}$ are believed to be important for extracellular detoxification of O_2^- such as produced by NADPH oxidase in macrophages. $SodC_I$ has been reported to be important during an infection of macrophages but there are conflicting reports on whether $SodC_{II}$ provides a role in virulence (Uzzau *et al.* 2002, Golubeva and Slauch 2006).

The detoxification of superoxide to hydrogen peroxide alleviates the threat of superoxide but hydrogen peroxide is readily able to produce hydroxyl radicals in the presence of a redox metal. *Salmonella* has six enzymes: AphC, BtuE, KatE, KatG, KatN and TsaA, capable of detoxifying hydrogen peroxide into water and oxygen as shown in the equation below.

$2H_2O_2 \rightarrow 2H_2O + O_2$

Again the redundancy of enzymes capable of detoxifying hydrogen peroxide emphasises the importance of its efficient removal. KatE, KatG and KatN are catalase enzymes that are the main mechanisms of hydrogen peroxide detoxification. Catalase does not become saturated by hydrogen peroxide at any concentration highlighting the efficiency of the enzyme (Lledias *et al.* 1998). KatG is located within the periplasm whereas KatE and KatN are located in the cytosol. As seen with the Sod enzymes, *Salmonella* therefore has a two layered detoxification system split between the periplasm and cytoplasm capable of detoxifying both superoxide and hydrogen peroxide. *Salmonella* also contains two cytoplasmic alkyl hydroperoxide reductases: AphC and TsaA that are also capable of detoxifying hydrogen peroxide (Mehta *et al.* 2007). Hebrard *et al.* (2009) identified that S. Typhimurium is only susceptible to hydrogen peroxide when a penta-mutant for: *katG*, *katE*, *katN*, *aphC* and *tsaA* was created. The penta-mutant could not grow in low concentrations of hydrogen peroxide highlighting the importance of the catalases and alkyl hydroperoxide reductases in reactive oxygen species resistance. An overview of reactive oxygen species detoxification in *Salmonella* is shown in figure 1.4. The intracellular presence of iron chelators salmochellin and enterobactin within S. Typhimurium provides reactive oxygen species protection independent of iron levels, although the mechanism of protection remains unknown (Achard *et al.* 2013). In the eventuality of reactive oxygen species damaging DNA the *rec* DNA repair system is upregulated. A *rec* system mutant is attenuated during macrophage infection unless reactive oxygen species damage is inhibited (Buchmeier *et al.* 1993).

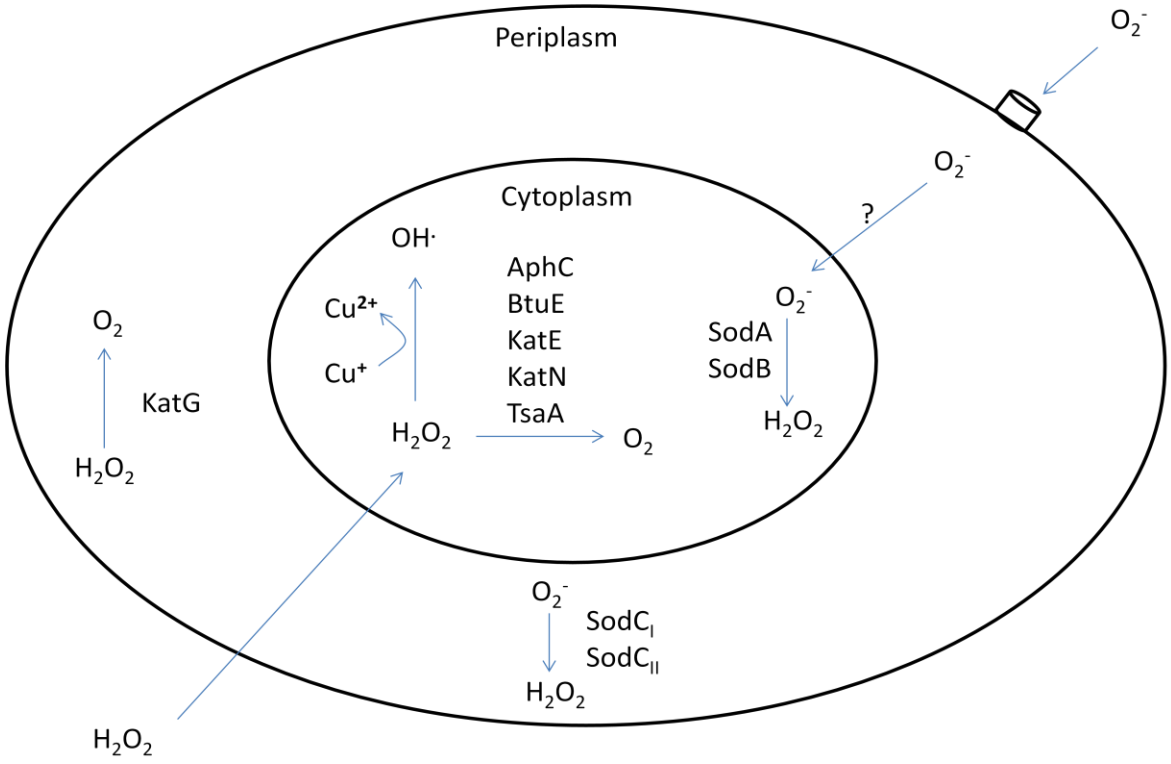


Figure 1.4 Reactive oxygen species detoxification within *Salmonella*

Hydrogen peroxide (H_2O_2) is capable of passively diffusing across the outer membrane where it can be converted into water and oxygen by catalase, KatG. Alternatively hydrogen peroxide can diffuse across the cytoplasmic membrane where it is detoxified by catalases KatN or KatE, alkyl hydroperoxide reductases AphC and TsaA or by glutathione peroxidase BtuE. If hydrogen peroxide is not removed or detoxified Fenton chemistry could occur between copper or another redox agent generating highly antimicrobial hydroxyl radicals ($OH^{\cdot}$). Superoxide (O_2^-) enters the periplasm through porins in the outer membrane of *Salmonella*. Superoxide is detoxified by super oxide dismutase $SodC_I$ and $SodC_{II}$ in the periplasm. It is currently unknown how superoxide enters the cytosol, but superoxide that does is detoxified by SodA and SodB.

1.6.3 Reactive nitrogen species

Reactive nitrogen species are nitrogen based ions or radicals with either one or two unpaired electron(s), enabling the nitrogen species to initiate a chemical reaction as a nucleophile. Humans possess three nitric oxide synthases (NOS): inducible nitric oxide synthase (iNOS), endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS). All NOS enzymes catalyse the production of nitric oxide from L-arginine and oxygen as shown in equation below.

L-arginine and oxygen + NADPH → N-hydroxy-L-arginine + NADPH → L-citruline and nitric oxide

The conversion of arginine and oxygen to citruline and nitric oxide is a five electron process with the formation of an intermediate molecule N-hydroxy-L-arginine (Stuehr *et al.* 1999). eNOS maintains vascular tone by the release of nitric oxide within blood vessels and nNOS utilises nitric oxide as a signalling molecule in the nervous system (Bolotina *et al.* 1994, Garthwaite *et al.* 1988). Inducible nitric oxide synthase (iNOS) as its name suggests is inducible, and thus is only expressed when stimulated. iNOS produces nitric oxide to inhibit bacterial growth within phagocytes. The inflammatory cytokines IL-1, TNF-α and IFN-γ upregulate the expression and production of iNOS and nitric oxide (Possel *et al.* 2000).

1.6.3.1 Regulation of iNOS

iNOS has a TATA box upstream of its promoter that is capable of binding multiple transcription factors including: Interferon regulatory factor 1 (IRF-1), nuclear factor-κB (Nf-κB), IFN-α-stimulated response element, tumour necrosis factor element and activating protein (Xie *et al.* 1993). The two primary pathways of iNOS activation are via IRF-1 by IFN-γ stimulation and Nf-κB from either LPS or TNF-α stimulation (Xie *et al.* 1994). iNOS is also negatively regulated by TGF-β which down regulates iNOS expression (Berg *et al.* 2007, Pullamsetti *et al.* 2006). Nitric oxide is capable of reversibly binding to the haem group within iNOS inhibiting further nitric oxide synthesis. Nitric oxide can also inhibit the addition of the haem group to apo-protein of iNOS preventing iNOS dimerisation and function (Albakri and Stuehr 1996).

1.6.3.2 Nitric oxide antimicrobial pathology

Upon synthesis iNOS is located within vesicles present within the cytosol of macrophages (Miller *et al.* 2004). Nitric oxide even though a free radical is relatively stable, capable of travelling up to 300 μm distances within a cell prior to reacting with a suitable target (Lancaster 1997). The free electron present on the nitrogen atom occupies a π-antibonding orbital around the entire molecule rather than remaining localised to the nitrogen atom. The dissociation of the electron from the nitrogen atom reduces the repulsion of electrons surrounding the nitrogen atom giving greater stability of the nitric oxide radical. The ability of nitric oxide to travel across large distances within a cell is also due to its hydrophilic nature enabling nitric oxide to cross lipid membranes. However iNOS does not need to localise to

phagocytosed endosomes for internal pathogens to come into contact with nitric oxide as such inhibition of iNOS localisation can affect the concentration of nitric oxide a phagocytosed pathogen encounters.

Reactive nitrogen species directly target lipids, proteins and to a lesser extent DNA. Not all reactive nitrogen species can damage DNA; dinitrogen trioxide (N_2O_3) can attack DNA causing deamination of a nucleotide. Nitric oxide has a weak damaging effect on DNA but can inhibit DNA replication. For example nitric oxide can displace zinc within zinc finger motifs' present in DNA polymerase (Schapiro *et al.* 2003). The ability of reactive nitrogen species to interact with metalloproteins is further discussed later (section 1.6.3.4). $^{\cdot}NO_2$ is currently the only know reactive nitrogen species capable of initiating lipid peroxidation, altering bacterial membrane fluidity, and can lead to a peroxide chain reaction as previously described (section 1.6.1). Reactive nitrogen species can also modify a variety of amides through nitrosation, nitrosylation and nitration that will be explained later (refer to section 1.6.3.3, Lui *et al.* 1998). An example of reactive nitrogen species inhibiting protein function is inhibition of respiration by binding to cytochrome *c* oxidase preventing its ability to reduce oxygen (Torres *et al.* 1995).

The importance of reactive nitrogen species in preventing internal bacterial pathogens has been highlighted in iNOS knockout mice that have increased susceptibility to *L. monocytogenes* and S. Typhimurium (Nathan and Shiloh 2000). Nitric oxide can inhibit the transcription of SPI2 virulence genes and PhoP regulated genes within S. Typhimurium if a threshold of nitric oxide is present. Repression of SPI2 and PhoP is seen within LPS or IFN-γ activated macrophages, but not within non-activated macrophages (Bourret *et al.* 2009).

The first reactive nitrogen species *Salmonella* encounters is in the stomach. Nitrate ingested from food is converted into nitrite by commensal oral flora in the mouth that is converted into acidified sodium nitrite (ASN) under the acidic conditions of the stomach (Pinheiro *et al.* 2012). ASN is a reactive nitrogen species generator exposing ingested *Salmonella* to reactive nitrogen species prior to cell invasion (Benjamin *et al.* 1994). Intracellular reactive nitrogen species are synthesised in response to cytokine stimulation whereas reactive oxygen species are pre-synthesised awaiting recognition of a foreign pathogen. *In vitro* experiments indentified that reactive nitrogen species are not produced by macrophages until approximately 8 hours after infection (Eriksson *et al.* 2003). The primary function of reactive nitrogen species is to inhibit further replication of the intracellular pathogen. Infection experiments with cell line macrophages and primary macrophages exhibit replication of internal *Salmonella* upon iNOS inhibition (Mastroeni *et al.* 2001). This is also seen for other intracellular pathogens including: *Chlamydophilia pneumonia* and *M. tuberculosis* (Carratelli

et al. 2005, Aston *et al.* 1998). Reactive nitrogen species have also been shown to inhibit viral infection for example; Coxsackie virus and Dengue virus (Zaragoza *et al.* 1997, Charnsilpa *et al.* 2005).

1.6.3.3 Nitrosylation, nitrosation and nitration

Reactive nitrogen species can initiate; nitrosylation, nitrosation and nitration of ligands. Nitrosylation is the addition of a nitric oxide derived radical $NO^{\cdot}$ or NO^{+} that forms a RNO group. Nitrosation is the direct donation of a nitric oxide or nitric dioxide radical from a compound forming a RNO group. Nitration is the addition of NO_2^{-} or NO_2^{+} to an amide. Reactive nitrogen species preferentially target sulphur ligands, the nitrosylation of a sulphur group is termed S-nitrosylation and nitrosation termed S-nitrosation. Nitrosylation and nitrosation provide a storage of reactive nitrogen species radicals that are capable of undergoing denitroslyation or denitrosation due to the labile properties of the covalent bond formed by NO groups (Vannin *et al.* 1997). The detoxification of nitrosylation and nitrosation requires a different mechanism of action to nitric oxide, although overlap in nitric oxide detoxification system and RNO detoxification has been identified. Hmp and NorV have been identified to provide denitrosylation action in S. Typhimurium and *N. Meningitidis*, respectively (Crawford and Goldberg 1998). S-nitrosoglutathione reductase is capable of detoxifying S-nitrosylation and is conserved across numerous species from humans to bacteria (Lui *et al.* 2001). The detoxification of nitrosylation and nitrosation groups is an expanding field and its importance in virulence is still under investigation.

1.6.3.4 Nitric oxide interactions with metals

Nitric oxide is capable of interacting with a variety of metal atoms, in particular iron. The binding of nitric oxide to iron-sulphur clusters inhibits protein function although this only occurs in enzymes that have an open pathway to the Fe-S cluster such as aconitase (Copper 1999). In addition to iron, nitric oxide is capable of displacing a variety of other metals from bound ligands, in various organisms with some examples listed in table 1.1

Table 1.1 Examples of metalloproteins susceptible to de-metallation by reactive nitrogen species

Organism	Metal	Protein
M. tuberculosis	Cu	Metallothionine
E. coli	Zn	DNA polymerase
Mice	Cd	Metallothionine

(Pearce *et al.* 2000, Binnet *et al.* 2002 and Gold *et al.* 2008)

1.6.4 *Salmonella* detoxification of reactive nitrogen species

1.6.4.1 Aerobic detoxification of reactive nitrogen species

Under aerobic conditions *Salmonella* detoxifies nitric oxide through its flavoheamoglobin (Hmp) that reduces nitric oxide into a nitrate ion (NO_3^-, Crawford and Goldberg 1998). Hmp is an iron containing metalloprotein that was discovered within *E. coli* (Vasudevan *et al.* 1990). Hmp contains a haem group within its C-terminal domain which is both the site of nitric oxide binding and recipient of electrons to catalyse reduction. Expression of *hmp* is regulated by the nitric oxide sensitive transcription factor NsrR. NsrR negatively regulates the expression of *hmp* by binding upstream of the *hmp* gene and inhibiting transcription. In the presence of nitric oxide a conserved iron-sulphur cluster [2Fe–2S] within NsrR is nitrosylated which inhibits its DNA binding capabilities removing NsrR repression and enabling *hmp* expression (Tucker *et al.* 2008). NsrR is a conserved regulator present in several intracellular pathogens including: *M. tuberculosis*, *E. coli* and *Neisseria meningitidis* (Bodenmiller and Spiro 2006, Rock *et al.* 2007). During S. Typhimurium infections of macrophages *hmp* is expressed indicating its role in detoxifying nitric oxide produced by iNOS. An *nsrR* mutant does not have any reduced tolerance to reactive nitrogen species but has increased killing within macrophages due to over expression of Hmp that has been implicated in generating reactive oxygen species (Gilberthorpe *et al.* 2007). In the absence of nitric oxide, Hmp can catalyse electron donation to oxygen, cytochrome *c* and several iron containing compounds generating potentially harmful products (Vausedevan *et al.* 1995, Andrews *et al.* 1992).

1.6.4.2 Anaerobic detoxification of reactive nitrogen species

Under anaerobic conditions reactive nitrogen species detoxification within *Salmonella* is performed by the flavorubedoxin, NorV and the cytochrome *c* nitrite reductase, NrfA. NorV functions with the aid of NorW to remove nitric oxide within *Salmonella* under anaerobic conditions. NorV catalyses the conversion of nitric oxide into nitrous oxide (N_2O). NorW is a flavoprotein that reduces NorV after catalysing the conversion of nitric oxide into nitrous oxide enabling NorV to reduce another molecule of nitric oxide. The expression of *norVW* is regulated by the transcription factor NorR that activates transcription in the presence of nitric oxide donors and S-nitrosoglutathione (GSNO, Flately *et al.* 2005). NorR senses nitric oxide through a mono-nitrosyl iron group that stimulates activation of an ATP hydrolysing core which subsequently stimulates σ_{54} driven RNA polymerase (D'Autreaux *et al.* 2005).

NrfA is a cytochrome oxidase that is conserved across many enteric pathogens and converts nitrite (NO_2^-) or $NO^\cdot$ into ammonium (NH_4^+) under anaerobic or microaerobic conditions. NrfA detoxifies $NO^\cdot$ into NH_4^+ with a similar rate of ~840 $NO^\cdot$ molecules s^{-1}, compared to 700 NO_3^- molecules s^{-1} (van Wonderen *et al.* 2008). NrfA is positively regulated by anaerobic transcription regulator FNR (fumarate nitrate reduction transcription regulator). FNR has four conserved cysteine residues of; 20, 23, 29 and 122 that provide detection of oxygen via a [4Fe-4S] cluster that under anaerobic conditions dimerises (Sharrocks *et al.* 1990, Khoroshilova *et al.* 1995). Dimerisation is key to binding of DNA and upregulation of gene expression. Phosphorylated nitrate and nitrite detecting protein NarL or NarP can bind with FNR to further activate transcription. NarL is phosphorylated in presence of nitrate whereas NarP is phosphorylated in the presence of nitrate and nitrite (Darwin and Stewart 1995). Within *E. coli nrfA* is negatively regulated by NsrR but experiments have not yet been performed to determine whether or not this is also the cause within S. Typhimurium (Filenko *et al.* 2007). NrfA has been identified to be most active under an acidic pH leading to the hypothesis that it is required during reactive nitrogen species exposure in the stomach (van Wonderen *et al.* 2008). NrfA is located in the periplasm of *Salmonella* and consequently is thought to be the first line of defence against reactive nitrogen species, if any reactive nitrogen species then progress to the cytosol NorVW and Hmp are present to remove the reactive nitrogen species threat depending on the availability of oxygen. Hmp can also provide nitric oxide detoxification under anaerobic conditions converting $NO^\cdot$ into N_2O but is significantly slower than its aerobic detoxification of nitric oxide, an estimated 100 fold reduced nitric oxide turnover rate (Mills *et al.* 2001). A single mutant for either *norV* or *nrfA* does not exhibit sensitivity to reactive nitrogen species under anaerobic conditions. However

ΔnorV/ΔnrfA strain is sensitive to reactive nitrogen species under anaerobic conditions highlighting the ability of NorV and NrfA to functionally replace the other (Mills *et al.* 2008).

A summation of the reactive nitrogen species detoxification pathways within *Salmonella* is shown in figure 1.5

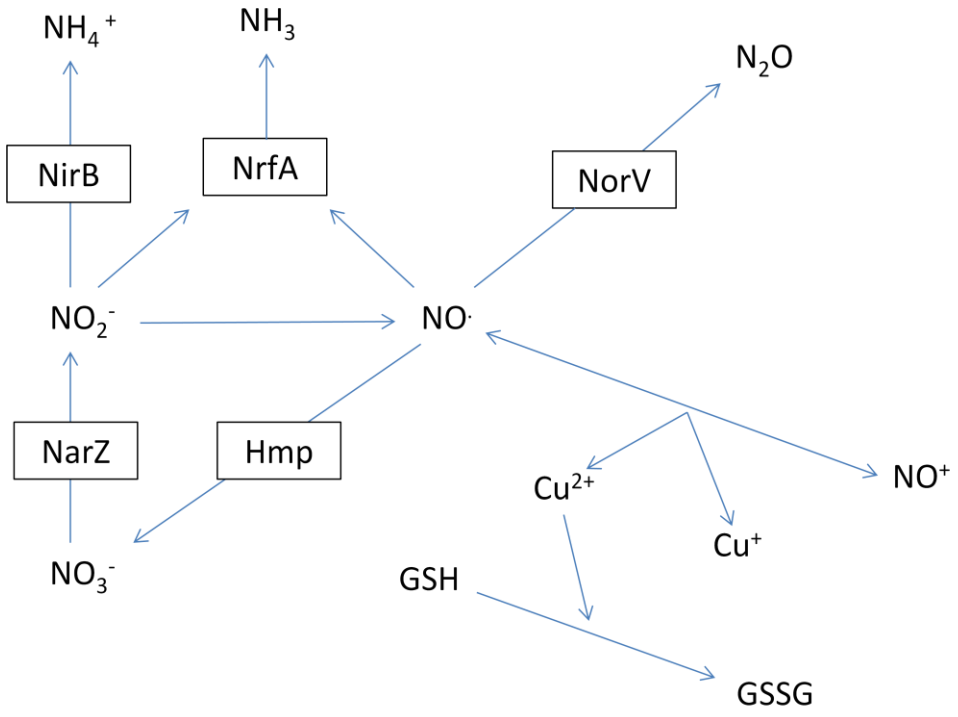


Figure 1.5 Reactive nitrogen species detoxification in *Salmonella*

Under aerobic conditions Hmp converts nitric oxide into nitrate. Under anaerobic conditions NorV converts nitric oxide into nitrous oxide and NrfA converts nitric oxide and nitrite into ammonia. Nitrate reductase NarZ and nitrite reductase NirB function under both aerobic and anaerobic conditions converting nitrate to nitrite and nitrite to ammonium respectively. The presence of copper can oxidise nitric oxide into a nitrosonium ion. Although, copper can be bound by glutathione inhibiting its generation of nitrosonium ions.

1.6.4.3 Reactive nitrogen species repair and avoidance genes

Upon reactive nitrogen species damage, *ytfE* is highly upregulated, and encodes an iron sulphur repair enzyme. A *ytfE* mutant exposed to nitric oxide does not have functional aconitase B and fumarate A enzymes but upon addition of purified YtfE protein to *ytfE* mutant cells, aconitase and fumarate enzyme functionality is restored (Justino *et al.* 2007).

M. tuberculosis and S. Typhimurium inhibit the co-localisation of iNOS into the *Mycobacterium* containing vacuole and SCV, respectively, reducing exposure to nitric oxide (Miller *et al.* 2004 and Chakavortty *et al.* 2002).

1.6.5 Peroxynitrite

Despite reactive oxygen species and reactive nitrogen species having sequential roles in antimicrobial defence their combination to form peroxynitrite has a key role in the antimicrobial activities of macrophages (Beckman *et al.* 1992). Peroxynitrite is a strong oxidising agent with greater antimicrobial potency than its constituents alone. Peroxynitrite is capable of initiating damage to DNA, lipids and proteins. Peroxynitrite is transported through ion channels in its anionic form ($ONOO^-$) or in its conjugate acid state (ONOOH) by passive diffusion (Romero *et al.* 1999). Peroxynitrite readily reacts with carbon dioxide to form $ONOOCO^-_2$ which is readily capable of oxidisation and nitrosylation of proteins and lipids (Denicola *et al.* 1996). Exposure of S. Typhimurium to peroxynitrite gave an upregulation of oxidative damage response genes: *katG*, and *ahpC* suggesting the peroxidation of macromolecules within S. Typhimurium (McLean *et al.* 2010). As previously described *Salmonella* contains two alkyl hydroperoxide reductases AphC and TsaA. An S. Typhimurium double mutant for *aphC* and *tsaA* has increased sensitivity to peroxynitrite (Bryk *et al.* 2000). However it is doubtful that within macrophages *Salmonella* is exposed to high levels of peroxynitrite due to SPI2 virulence factors inhibiting the localisation of NADPH-oxidase and iNOS to the SCV (Chakavortty *et al.* 2002 and Vazquez-Torres *et al.* 2000).

Peroxynitrite can perform oxidative damage of both a nucleotide base and sugar backbone. Guanine has the lowest reductive potential of the four nucleotide bases and consequently is targeted by peroxynitrite. Peroxynitrite induced damage can result in transversion of the guanine base to thymine or cytosine within *E. coli* giving significant mutagenic effects (Henderson *et al.* 2002). Peroxynitrite can also damage the sugar backbone of DNA by extracting a hydrogen ion from a deoxyribose sugar and addition of an oxygen molecule results in the breaking of the DNA strand (Szabo and Hiroshi 1997).

Peroxynitrite preferentially targets sulphur amino acids; cysteine and methionine, but can also affect tryptophan (Alvarez *et al.* 1998). Peroxynitrite can damage thiol amines by removing a H^+ ion from the thiol group leading to the addition of a hydroxyl group forming sulfenic acid. In the presence of another sulfenic acid amide a disulphide bond is formed between them modifying protein structure. Peroxynitrite can also attack cysteine to form a thiyl radical ($RS^{\cdot}$) which can then react with oxygen forming a reactive oxygen species or can

bind a nitrosium ion and form a nitrosothiol group (Balazy *et al.* 1998). Peroxynitrite can directly attack other amides such as histidine and tyrosine; or products from the breakdown of peroxynitrite including; $OH^{\cdot}$, $NO_2^{\cdot}$ and CO_3^{-} which can initiate damage to multiple macromolecules also (Bonini *et al.* 1999). Both oxy and nitro derivatives of: histidine, phenylalanine, tyrosine, tryptophan amines have been identified as secondary peroxynitrite radicals (Alvarez *et al.* 1998). As previously stated peroxynitrite can initiate lipid peroxidation by the removal of a H^+ ion from mono or poly unsaturated lipid or lipoprotein (Richter 1987).

1.6.6 Overview of reactive species within *Salmonella*

Salmonella is exposed to a variety of reactive species during intracellular survival. Reactive oxygen species and reactive nitrogen species form a network of reactive species capable of damaging different targets within *Salmonella* as shown in figure 1.6

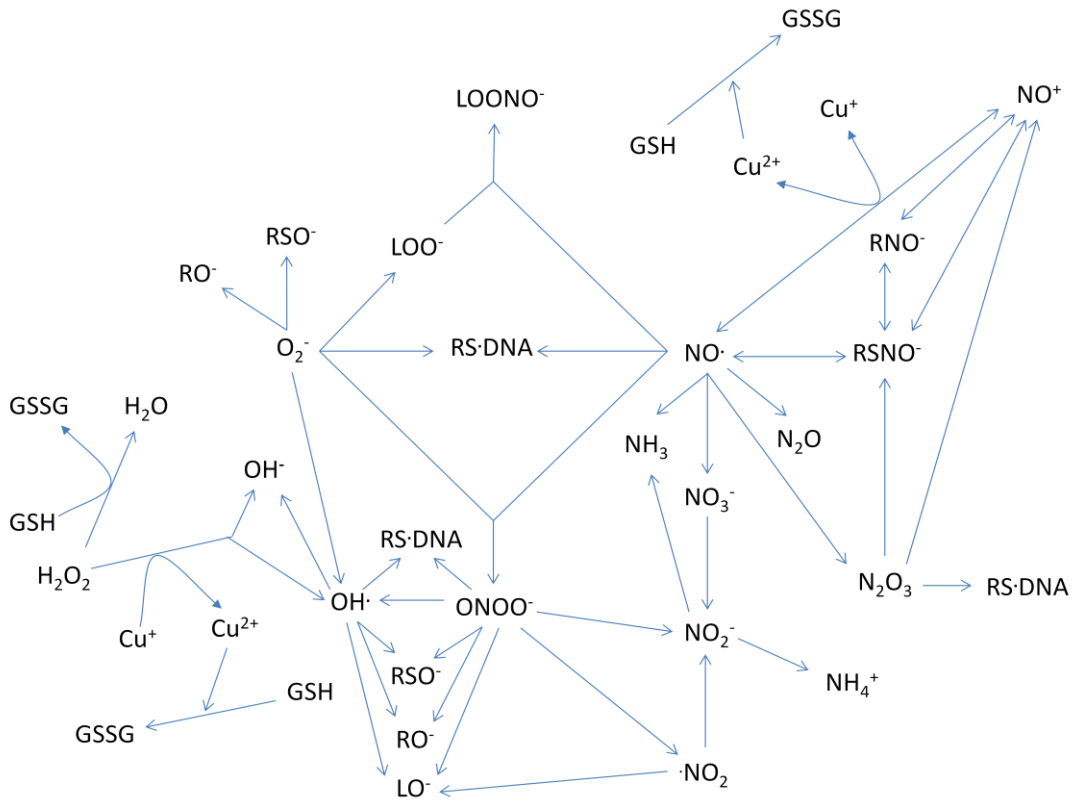


Figure 1.6 Overview of reactive species reactions within *Salmonella*

The addition of hydrogen peroxide (H_2O_2) or the synthesis of superoxide (O_2^-) and nitric oxide ($NO^·$) can result in a variety of reactive species. Superoxide can oxidise proteins of an amide group (RO^-) or thiol group (RSO^-) or initiate lipid peroxidation (LOO^-). Hydrogen peroxide can be converted into hydroxyl radicals ($OH^·$) through Fenton chemistry driven by copper or redox active metals. Copper itself is buffered by glutathione (GSH) reducing its ability to catalyse Fenton chemistry. Glutathione can also breakdown hydrogen peroxide in the presence of glutathione peroxidase. Hydroxyl radicals and peroxynitrite ($ONOO^-$) are the most potent oxidising reactive species capable of causing significant damage to *Salmonella*. Hydroxyl radicals and peroxynitrite affect similar ligands to superoxide but cause significantly greater damage. Peroxynitrite if combines with carbon dioxide breakdowns to form nitrite radicals ($^·NO_2$) that can initiate lipid peroxidation. Nitric oxide ($NO^·$) preferentially targets thiol groups giving nitrosylated amides (RSNO). Nitric oxide can be oxidised to a nitrosonium ion (NO^+) in the presence of copper which can bind to amine groups causing nitrosation of an amide (RNO) or nitrosylate an amide. Nitric oxide can bind DNA and lipids but only causes minimal damage. Nitric oxide can combine with superoxide to form peroxynitrite. Nitric oxide is detoxified to less reactive forms of nitrogen based molecules by Hmp to nitrate (NO_3^-), by NorV to nitrous oxide (N_2O) or alternatively converts nitrite (NO_2^-) to ammonia (NH_3) and NrfA coverts nitric oxide to ammonium (NH_4^+) depending on availability of oxygen. Nitrate reductase reduces nitrate to nitrite and nitrite reductase can reduce nitrite to ammonia. Nitric oxide can form dinitrogen trioxide (N_2O_3) that readily forms nitrosoium ions enabling nitrosation and nitrosylation. Dinitrogen trioxide has also been observed to damage DNA.

1.7 *Salmonella* cytoplasmic regulatory features

1.7.1 Maintaining a reduced cytoplasm

Bacteria maintain a reduced cytosol to control protein folding. In a reduced environment few disulphide bonds can form, whereas in an oxidised environment disulphide bonds are prevalent. The unintentional formation of disulphide bonds can prevent correct folding and function of a protein. Within *Salmonella* two pathways involving the small proteins glutathione and thioredoxin ($TR\text{-}SH_2$) maintain a reduced cytoplasmic redox potential. Both glutathione and thioredoxin ($TR\text{-}S_2$) oxidised states can be re-cycled to the reduced form by utilising the reduction potential of NADPH by the actions of glutathione reductase and thioredoxin reductase, respectively, as shown in the equation below (Bessette *et al.* 1999).

$$GSSG + NADPH + H^+ \rightarrow 2GSH + 2NADP^+$$

$$TR\text{-}S_2 + NADPH + H^+ \rightarrow TR\text{-}SH_2 + 2NADP^+$$

The ratio of glutathione to oxidisied glutathione and thioredoxin to oxidised thioreodoxin is an important. It is suggested that the oxidised forms of glutathione and thioredoxin do not exceed 5% of the total reducing agent. The ability of reduced glutathione and thioredoxin to respond rapidly to an environmental stress is important in maintaining multiple homeostasis pathways for *Salmonella*.

1.7.2 Glutathione

Glutathione has been identified to provide a role in metal homeostasis, reactive species stress, pH regulation and osmotic stress (Chesney *et al.* 1996, Song *et al.* 2013). *E. coli* is estimated to maintain an approximately 5 mM concentration of glutathione within the cytoplasm and a reported lesser concentration in the periplasm (Helbig *et al.* 2008). Glutathione is produced in a two step process; initially glutamate and cysteine are converted into L-γ-glutamylcystenine by glutamate-cysteine ligase. L-γ-glutamylcystenine and glycine are converted into glutathione by glutamine synthase (GshB). Two molecules of glutathione can be oxidised or bind an oxidising agent such as Cu^+ and form a molecule of oxidised disulphide glutathione, GSSG, reducing the oxidising agent in the process. Oxidised disulphide glutathione (GSSG) can be reduced back to glutathione by the actions of glutathione reductase (Gor) that utilises electrons from the $NADP^+$/NADPH reductant pool.

A mutant for glutathione production within *E. coli* exhibited a slight reduction in tolerance to Zn and Cu (Helbig *et al.* 2008). The combination of a glutathione mutant (*ΔgshB*) with a mutation for *zntA* (Zn cytosolic exporter) or c*opA* (copper cytosolic exporter) gave significantly reduced tolerance to zinc and copper respectively (Helbig *et al.* 2008). The additive nature of a glutathione mutation to the primary exporter for copper (*copA*) or zinc (*zntA*) in *E. coli* supports the premise that when the cytosol is overloaded with metal ions glutathione can bind metal ions to prevent harmful effects free metals can have on a cell, as previous discussed (section 1.11.3). Therefore the role of glutathione in metal detoxification may inhibit harmful effects that would transpire when a metal detoxification system is compromised or whilst metal responsive operons' are expressed and functional. Although if a large proportion of cellular glutathione is required to bind metal within the cytosol it will compromise the reducing potential of the cell. Consequently the cell could be more susceptible to other antimicrobials that glutathione contributes to the detoxification of including reactive oxygen and nitrogen species. It is unclear if glutathione provides a role in aiding export of metals and is a consideration for future research.

Glutathione also provides a role in the detoxification of reactive oxygen and reactive nitrogen species. Glutathione has been identified to contribute to the detoxification of hydrogen peroxide and peroxynitrite through the actions of glutathione peroxidase (Chesney *et al.* 1996, Arenas *et al.* 2010). A glutathione mutant has been reported to exhibit increased susceptibility to reactive oxygen species in *E. coli* (Goswami *et al.* 2006). Glutathione provides a role in the detoxification of reactive nitrogen species. A *Salmonella* glutathione mutant has increased sensitivity to reactive nitrogen species than a wild-type strain; and a glutathione mutation is additive to a *hmp* mutant (Song *et al.* 2013).

1.8 Copper

Copper is a transition metal that typically has an oxidation state of Cu^{+} or Cu^{2+}. Copper is an essential micronutrient for all kingdoms of life (Harris 2001). The ability to switch oxidation states between Cu^{+} and Cu^{2+}, to provide or accept an electron, has led to its incorporation within catalytic sites for several enzymes most of which are extra-cytoplasmic. Examples of copper containing proteins are listed in table 1.2.

Table 1.2 Examples of cuproproteins

Species	Cuproprotein	Function
Mammalian, prokaryotic and plants	Cytochrome *c* oxidase	Reduces oxygen into water as the final electron acceptor of the electron transport chain during respiration
Mammalian, prokaryotic and plants	Cu, Zn superoxide dismutase	Catalyses the conversion of superoxide into hydrogen peroxide
Mammalian	Dopamine β-hydroxlase	Converts dopamine into norepinehrine, a neurotransmitter
Plants	Plastocyanin	Transfers electrons between photosystem I and II during photosynthesis
Mammalian	Lysyl oxidase	Oxidises lysine amines to the aldehyde allysine that impacts the formation of extracellular matrix
Animal	Haemocyanin	Carries oxygen as an alternative to haemoglobin
Mammalian and prokaryotic	Laccase	Detoxifies reactive oxygen species and Cu^{+} into Cu^{2+}

(Wenk and Suzuki 1983, Merchant and Bogorad 1986, Rucker *et al.* 1998, Bauerfeind *et al.* 1997)

1.8.1 Importance of copper homeostasis

As previously stated copper provides several essential actions within numerous organisms but regulation of intracellular copper levels are essential. Copper is highly reactive capable of binding to numerous ligands within a cell, disrupting their function and having harmful

effects upon the cell. Copper is ranked highest within the Irving-Williams series that quantifies divalent metal complex stabilities as shown below.

$Mn^{2+} < Fe^{2+} < Co^{2+} < Cu^{2+} < Cu^{+} > Zn^{2+}$

(Irving and Willams 1948, Foster and Robinson 2011)

Thus, copper forms complexes with greater stability than other divalent metal cations. Consequently, if unregulated, copper is capable of disrupting the binding of other metal complexes, particularly iron-sulphur clusters, and can cause damage due to catalysis of Fenton chemistry in the presence of H_2O (Fenton 1894, Macomber and Imlay 2009).

1.9 Mammalian copper homeostasis

1.9.1 Copper uptake and distribution

Copper is absorbed within the gut from dietary intake, gut epithelial cells uptake copper through the copper transporter Ctr1 which requires the reduction of Cu^{2+} into Cu^{+} for uptake. A reductase adjacent to Ctr1 reduces copper to Cu^{+} enabling uptake. Analysis of copper uptake in *S. cerevisiae* revealed the requirement for a copper reductase present on the surface of its membrane to reduce copper and enable uptake through Ctr1 (Rees and Thiele 2007). Depending on cellular copper concentration, the location of Ctr1 changes between membrane associated and vesicle bound. At low copper concentrations Ctr1 is membrane associated to facilitate copper uptake, whereas at high copper concentrations Ctr1 is vesicle bound and degraded (Petris et al. 2003). A *ctr1* knockout mouse has reduced intracellular copper levels and severe growth defects but copper is uptaken, indicating Ctr1 is not the only transporter capable of uptake, but is required for normal growth (Nose *et al.* 2006). Intracellular copper is bound upon entry by the copper chaperone Atox1, which shuttles copper to the copper transporting P_{1B}-type ATPase ATP-7a at the Golgi apparatus for incorporation into cuproproteins (Kim *et al.* 2012). Other copper chaperones include copper chaperone for superoxide dismutase (CCS) and copper chaperone for cytochrome *c* oxidase (Cox17) which supplies copper to the mitochondria (Casareno *et al.* 1998, Palumaa *et al.* 2004). A knockout mutant for *atox1* is unable to survive, death occurs before birth highlighting the importance of Atox1 (Hamza *et al.* 2003).

Copper absorbed by the gut is transported to the liver and converted into bile or is released through the copper transporter ATP-7b into the blood supply in a complexed form with; ceruplasmin, albumin or transcuprein (Lui *et al.* 2007). Copper is then transported to other

organs including the: brain, heart and kidneys. The storage of copper within mammalian cells remains unknown. Studies within *S. cerevisiae* identified that copper is stored within a vacuole organelle and a Ctr1 homologue, Ctr2, is involved in the mobilisation of copper stores within the vacuole and is a possible mechanism in mammalian cells (Rees *et al.* 2002).

The inability to uptake and re-distribute copper results in physiological disorders. Mutations of *atp-7a* that render the transporter unable to transport copper result in the clinical condition Menkes disease, that is characterised by copper accumulation within the gut epithelia due to inefficient export into the blood (Vulpe *et al.* 1993). Symptoms of Menkes disease include mental welfare implications, low body temperature, brittle kinky hair and osteoporosis (Menkes *et al.* 1962). Foetuses with Menkes disease are also highly at risk of premature birth. An *atp-7b* mutant unable to export copper experiences copper over-accumulation within the liver, kidneys and brain and is termed Wilson's disease (Bull *et al.* 1993). Wilson's disease effects include enlargement of the liver potentially progressing to acute liver failure and neuropsychiatric problems such as migraines, seizures and lack of coordination (Wilson *et al.* 1912). The significant health implications caused by Menkes and Wilsons disease highlights the importance of copper homeostasis in humans.

1.9.2 The role of copper in innate immune system

Copper provides antimicrobial actions including: catalysing Fenton chemistry, inhibiting iron-sulphur clusters and mis-metallation of metalloproteins. Initially it was believed that copper toxicity was primarily through Fenton chemistry, copper has also been identified to directly cause detrimental effects when unregulated within bacteria. Copper forms stable complexes and can consequently displace other metals that are lower in the Irving-Williams series (section 1.8.1). Copper has been identified to target iron sulphur clusters present on the external surface of proteins (Macomber *et al.* 2007). Copper is unable to target iron sulphur clusters concealed within a protein only those solvent accessible. An *E. coli* strain containing mutations of *copA*, *cusCFBA* and *cueO* is highly susceptible to copper and had inactivity of dehydratase enzymes in the presence of copper (Macomber *et al.* 2007). Dehydratase enzymes are involved in branch chain amino acid synthesis (leucine, isoleucine and valine). If unregulated copper can also bind to metal binding sites for non-cuprous metalloproteins inhibiting their function.

Copper provides a role within an immune response against intracellular pathogens, at sites of inflammation raised copper levels are detected (Beveridge *et al.* 1985). Mutation of copper

detoxification systems for various pathogens including: S. Typhimurium, *Candida albicans, M. tuberculosis, P. aeruginosa* and *E. coli*, results in reduced intracellular survival within macrophages identifying a copper mediated antimicrobial response by the innate immune system (Osman *et al.* 2010, Jones and Suttle 1981, Wolschendorf *et al.* 2011, Schwan *et al.* 2005, White *et al.* 2009).

Macrophages uptake copper from the external environment through Ctr1 with the aid of a group of cupric and ferric reductases called Steap proteins that reduce Cu^{2+} into Cu^{+} (Ohgami *et al.* 2006). Expression of Ctr1 increases upon bacterial infection of macrophages and expression is further exaggerated by the activation of macrophages prior to infection with LPS or IFN-γ (White *et al.* 2009, Achard *et al.* 2013). Once copper is uptaken it is bound by Atox1 that transports copper to ATP-7a that can localise to phagosome associated vesicles and potentially transport Cu^{+} into the phagosome (White *et al.* 2009). ATP-7a expression has also been reported to increase upon bacterial infection of macrophages, supporting the premise that copper provides an antimicrobial action against the internalised pathogen (White *et al.* 2009, Achard *et al.* 2013). RNA interference of *atp-7a* expression gave increased survival of *E. coli* internalised within macrophages, supporting the theory that ATP-7a directly pumps copper into the phagosome (White *et al.* 2009). The mechanism by which macrophages transport copper into the phagosome remains unclear.

1.10 Bacterial copper homeostasis

1.10.1 Copper uptake

Few bacteria have been identified to encode copper uptake transporters. *Enterococcus hirae* encodes a copper transporter, CopA, which is suggested to uptake copper under low copper levels (Odermatt *et al.* 1993). CopA has also been shown to interact with CopZ; a known metallochaperone that supplies copper to transcription regulator CopY, supporting the hypothesis that copper is being uptaken by CopA (Multhaup *et al.* 2001). *Listeria monocytogenes* contains a P_{1B}-type ATPase CtpA which has been suggested to import copper; *ctpA* mutants have lower internalised levels of copper (Francis and Thomas 1997). Also, *Pseudomonas aeruginosa* contains a CtpA homologue HmtA, a P_{1B}-type ATPase that transports copper and zinc (Lewinson *et al.* 2009). Cyanobacteria contain two P_{1B}-type ATPases, CtaA that associates with the outer membrane and is suggested to import copper and PacS that associates with the thylakoid membrane and imports copper into the thylakoids (Robinson and Winge 2010). However, Raimunda *et al.* (2011) reported contrasting results and suggest that a P_{1B}-type ATPase would be unable to import copper

and hence suggest: CtaA and CopA actually exclude copper at varying rates within their respective organisms.

Although the mechanism of copper uptake in S. Typhimurium and *E. coli* is unknown, outer membrane factors have been identified to influence copper uptake within *E. coli*. ComC has been identified to influence the permeability of copper to the outer membrane. In an *E. coli comC* mutant, increased levels of copper were detected in the periplasm and increased expression of the *cusCFBA* operon associated with copper efflux (Mermod *et al.* 2012). *Salmonella* encodes a homologue to ComC, hence the expression of *comC* could influence copper uptake by *Salmonella*. It is currently suggested that copper enters the cytosol of Gram negative bacteria through diffusion. It is thought Cu^{+} has increased ability to diffuse through the cytoplasmic membrane than Cu^{2+} due to its reduced polarity (Changela *et al.* 2003). To conclude copper uptake within prokaryotic organisms remains highly unknown and further research is required to determine the mechanism of copper uptake.

1.10.2 P_{1B}-type ATPases

The most common employed copper exporting mechanism involves P_{1B}-type ATPases, which are aptly named due to a conserved DKTGT sequence of which the aspartate residue becomes phosphorylated whilst functioning (Pedersen and Carafoli 1987). P_{1B}-type ATPase contain eight transmembrane helical loops forming the channel region of the protein with an ATP hydrolysing region present between helices 6 and 7 (Solioz and Vulpe 1996). One molecule of ATP is hydrolysed during the transport of one atom of metal. P_{1B}-type ATPases exhibit specificity to different metal ions and factors that contribute to this include metal binding sites within a cytoplasmic N-terminal region and a combination of transmembrane segments (Arguello *et al.* 2003). Several P_{1B}-type ATPases transport specificity identified based on these characteristics such as; b0013, b0022 and b0072 (Banci *et al.* 2002, Borrelly *et al.* 2004, Hou *et al.* 2001). Numerous copper P_{1B}-type ATPases contain a cysteine amine amine cysteine sequence (CXXC) within a cytoplasmic N-terminal region that binds copper. Indeed, the CXXC sequence is the site which a P_{1B}-type ATPase acquires copper from a copper chaperone by ligand exchange and can consequently be exported (Huffman and O'Halloran 2000).

Copper detoxification also involves the family of CBA proteins in Gram negative bacteria that contain three components; an inner membrane transporter (A), a membrane fusion protein (B) and an outer membrane exporter (C). CBA proteins are involved in the exportation of various substrates from the cytosol and periplasm to the external environment (Paulsen *et*

al. 2006). CBA proteins include resistance nodulation division family (RND) efflux systems such as the *cusCFBA* system present within *E. coli* that exports copper (section 1.14). CBA proteins utilise the proton motive force rather than ATP hydrolysis in facilitating copper ion movement (Nies 1995).

1.10.3 RND efflux systems

The Cus system is an example of an RND efflux system. RND systems export antibiotics and other harmful compounds from Gram negative bacteria. RND exporters have been categorised into seven families based on their exported agents that include macro and micro molecules such as; metals, antibiotics and liposaccharides (Tseng *et al.* 1999). Well established RND metal exporters include CusC and CnrC that export Cu^{+} / Ag^{+} and Co^{2+} / Ni^{2+}/ Cd^{2+} respectively (Franke *et al.* 2003, Schmidt and Schlegel 1994). RND efflux pumps are a type of CBA system consisting of an inner membrane translocase, a periplasmic membrane fusion protein and an outer membrane factor, as shown figure 1.7 (Thanabalu *et al.* 1998). TolC provides the outer membrane factor for a variety of RND efflux systems.

1.10.4 TolC

1.10.4.1 TolC structure and function

TolC is a trimer that contains two regions; a uniform β-barrel of 40 Å in length that spans the outer membrane and an α-barrel of 100 Å that extends into the periplasm, giving a 140 Å total size (Koronakis *et al.* 2000). The channel of TolC has a diameter of approximately 30 Å facilitating the transport of large agents such as antibiotics and toxins (Koronakis *et al.* 1997). The channel opening of the β-barrel region is unregulated and remains open, the α-barrel channel opening in the periplasm is tightly regulated to occlude the transport of micro and macro nutrients unless stimulated.

Two models have been suggested for the mechanism of export by TolC as part of an RND efflux system: a funnel and a switch mechanism as shown in figure 1.7. The switch mechanism is initiated by the binding and extraction of a transportable substrate from the cytosol by the cytoplasmic translocase. A membrane fusion protein is recruited to the periplasmic region of the cytoplasmic translocase and sequentially recruits TolC. Binding of the substrate to the membrane fusion protein facilitates a change in the conformation of

TolC's α-barrel channel, opening and transferring the substrate to TolC that actively exports the substrate across the outer membrane. The funnel mechanism extracts the substrate from within the periplasm. The membrane fusion protein binds the substrate then binds to a cytoplasmic translocase which then recruits TolC. TolC undergoes a conformational change to open its α-barrel channel upon binding the cytoplasmic translocase and membrane fusion protein complex, and TolC exports the agent across the outer membrane. Both transport mechanisms are dependent on each component of an RND efflux system undergoing a conformational change during transport of the substrate (Koronakis *et al.* 1998).

How specificity for RND efflux systems is dependent upon the efflux system analysed. TolC itself does not provide specificty for what it exports across the outer membrane of Gram negative bacteria. If the switch mechanism is believed to take place then the selective step is believed to occur within the cytosol. Selectivity could be achieved through binding sites on the innermembrane transporter or alternatively from the binding of a chaperone to the innermembrane transporter. Conversely, a transporter utilising a funnel mechanism exports from the periplasm, and selectivity is achieved in the periplasm as well. This can be through the binding to a periplasmic region of the innermembrane transporter or a periplasmic chaperone.

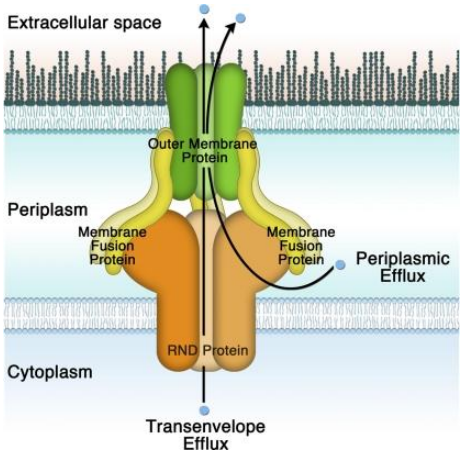


Figure 1.7 Methods of substrate efflux by RND efflux systems

RND efflux pumps are capable of switch (transenvelope) efflux extracting the substrate from the cytosol or funnel from within the periplasm and exporting the substrate across the outer membrane into the external environment. Taken from Kim *et al.* 2011.

1.10.4.2 The roles of TolC in bacteria

TolC acts as the outer membrane factor for several systems exporting a range of different compounds including: antibiotics, bile salts and metals (Fralick 1996, Baucheron *et al.* 2005, Giraud *et al.* 2000). The importance of TolC exportation is exhibited through the pleiotropic effects that happen to *tolC* mutants of various Gram negative bacterial species. TolC is capable of exporting antimicrobial compounds from within a bacterium, such as bile salts and antimicrobial peptides (polymyxin B or human beta defensin-1 peptides) (Warner and Levy 2010). TolC can also export virulence factors; *E. coli* and *Neisseria meningitidis* export active alpha-haemolysin through TolC (Vakharia *et al.* 2001, Kamal and Shafer 2010). TolC can also influence virulence by directly contributing to adhesion and survival; a *tolC* mutant has reduced adhesion and survival during infections of cell line or animal models with: *Legionella pneumophila*, S. Typhimurium and *Francisella tularensis* (Ferhat *et al.* 2009, Buckley *et al.* 2006, Gill *et al.* 2006). TolC also provides a role in maintaining a viable cytosol by the removal of metabolites. *E. coli* and *Sinorhizobium meliltoi tolC* mutants upregulate stress responses due to an inability to efficiently remove metabolites (Rosner and Martin 1999, Santos *et al.* 2010). The ability of TolC to influence: antimicrobial tolerance, gene expression, membrane stability, metabolite export and virulence, by its exportation of numerous substrates conveys the importance of TolC.

1.10.4.3 The roles of TolC in *Salmonella*

TolC is an important outer membrane factor for several drug efflux systems exporting antibiotics and antimicrobials outside of *S.* Typhimurium as shown within table 1.3. Several RND efflux systems have functional redundancy to remove antimicrobials. The inhibition of RND efflux systems is a current tatic utilised by pharmaceutical companies to combat the increase in antibiotic resistance.

TolC has also been implicated in contributing to *Salmonella* survival during infections, with a *tolC* mutant having reduced expression of 112 genes including SPI1 virulence factors (such as SipA, SipC and SopE) involved in uptake of *Salmonella* by a non-phagocytic cell (Buckley *et al.* 2006, Webber *et al.* 2009). A *tolC* mutant has reduced adhesion and survival in both macrophage cell line and mouse model infection experiments (Buckley *et al.* 2006, Nishino *et al.* 2006). TolC also has been identified to be involved in tolerance of copper and zinc in *S.* Typhimurium (Nishino *et al.* 2007).

GesABC and MdtAB have been identified to provide export of gold and copper/zinc from S. Typhimurium respectively (Nishino *et al.* 2007, Pontel *et al.* 2007). Although further research needs to be conducted to expand the metals tested to identify if either system is capable of exporting alternate metals. Its is lso currently unknown how the Ges and Mdt system interacts with pre-estabished metal detoxification systems and requires further research.

Table 1.3 RND family efflux system that function in association with TolC

Inner membrane translocase and membrane fusion proteins that associate with TolC	**Agent exported**
AcrAB	Bile salts, SDS, Triton, Multiple drugs
AcrAD	Bile salts, Novobiocin, SDS, Aminoglycosides
AcrEF	Multiple drugs
EmrAB	Novobiocin, Naladixic acid, SDS
GesAB	Gold, Multiple drugs, SDS
MdtAB	Novobiocin, Naladixic acid, SDS
MdsAB	Novobiocin, Crystal violet, SDS, Metheylene blue, Acriflavine
MdtAC	Novobiocin, Naladixic acid, SDS, cation metals
MacAB	Macrolides

(Horiyama *et al.* 2010, Sulavik *et al.* 2001, Conroy *et al.* 2010)

1.11 Incorporation of metals into metalloproteins

1.11.1 Metallochaperones

Metallochaperones are small metal binding proteins that deliver and exchange metal ions with an intended cellular target. Metallochaperones prevent the mis-metallation of metalloproteins by directly transferring a metal to a protein preventing adverse side reactions of metals en route (Robinson and Winge 2010).

As previously stated, copper is toxic when unregulated within a cell or bacterium and is consequently complexed rapidly upon entry. Copper chaperones ensure that copper is transported to the correct cellular location. Copper chaperones tend to not provide any resistance to copper, with an *atx1* mutant having no difference in copper tolerance within *S. cerevisiae* (Rae *et al.* 1999). Although, deletion of copper chaperones in combination with other copper homeostatic systems such as glutathione, can result in a drop in copper tolerance as seen within *Synechocystis Δatx1/ΔgshB* (Tottey *et al.* 2012). However, a *B. subtils copZ* mutant exhibits reduced growth in the presence of copper (Radford *et al.* 2003). Although copper chaperones do not provide a role in copper tolerance the binding of copper could potentially prevent harmful effects such as iron-sulphur cluster disruption (Tottey *et al.* 2012).

Multiple copper chaperones contain a conserved protein sequence of GXXCXXC, in which the two cysteine amides form a thiol bridge with a copper ion (Rosenzweig 2002). The binding properties of the CXXC region provide adequate retention of copper whilst capable of releasing copper once the correct ligand is bound. Copper chaperones have been identified to supply copper to numerous cellular components including; enzymes, transporters and transcription regulators. Copper chaperones have been identified in both the cytosol and periplasm for example Atx1 (CopZ), within the cytosol of several bacteria and eukaryotes and CusF within *E. coli* is present within the periplasm (Loftin *et al.* 2005).

Atx1 homologues have been reported in *H. pylori, Synechcystis, S. cerevisiae* and in *B. subtilis* and are also referred to as CopZ (O'Halloran 2000, Radford *et al.* 2003, Walker *et al.* 2002,). Atx1 (CopZ) can directly interact with P_{1B}-type ATPases through their N-terminal domain; a series on interactions occur between Atx1 and the N-terminal domain that enables the donation of copper (Rodriguez-Granillo *et al.* 2010). However, the N-terminal domain is not required for binding and transportation of copper by CopA in *Archaeoglobus fulgidus* (Mandal and Arguello 2003). Hence, N-terminal domain metal binding is not essential for copper transfer for export by P_{1B}-type ATPases as Atx1 can directly bind to transmembrane domains and donate copper (Mandal and Arguello 2003). Atx1 in cyanobacteria interacts and exchanges copper with two different P_{1B}-type ATPases; CtaA and PacS. CtaA is suggested to import copper into the cytosol which is bound by Atx1, which transports copper to PacS that imports copper into the thylakoids (Raimunda *et al.* 2011, Kanamaru *et al.*1994, Tottey *et al.* 2001). CopZ within *E. hirae* is important for interacting with the copper-responsive regulator CopY, transferring a Cu^{+} atom to CopY to displace Zn^{2+} which can relieve CopY repression of the *cop* operon (Cobine *et al.* 2002).

1.11.2 Protein folding regulation of metallation

The incorporation of a metal into a metalloprotein can be dependent on the location of protein folding. MncA and CucA (reviewed Waldon and Robinson 2009) are both periplasmic metalloproteins, CucA binds copper and MncA binds manganese. However, copper has a greater binding affinity for both non-metallated proteins than manganese, consistent with the Irving-Williams metal binding stability series (section 1.8.1). A direct study of the metalloproteins identified a 10,000 fold molar excess of manganese to copper was required to ensure MncA bound manganese and not copper. To achieve correct metallating MncA therefore undergoes protein folding within the cytosol (due to a 10,000 fold concentration difference between manganese and copper) and is exported via the twin arginine translocase pathway, for folded proteins. In contrast, CucA is transported via the general secretory pathway in its unfolded state into the periplasm where it receives copper and folds into its correct confirmation. The separation of MncA and CucA metallation steps thus ensures MncA has manganese bound before coming into contact with copper in the periplasm. Also the manganese site is buried within MncA, once manganese is bound and folded, whereas in an unfolded-state the manganese site is accessible and can be bound by copper. The folding of MncA in the cytosol removes spatial access of copper and gives a low off rate for manganese dissociating from its binding site thus allowing MncA to retain manganese even when exported to the periplasm.

1.11.3 Antimicrobial effects of metals

Metals can induce toxicity to a cell by numerous mechanisms including: protein dysfunction, reactive species production, membrane integrity compromised, genotoxicity and nutrient uptake inhibition. Metal toxicity has been reviewed well by Lemire *et al.* (2013) figure 1.8. Metalloproteins can bind numerous metals at their metal binding sites. It is through several levels of regulation in which an apo-protein is supplied with the correct metal to form a functional metaloprotein such as compartmentalised folding, chaperone transport of metal to the apo-metalloprotein or export/sequestering of metals. Divalent cation metal ions follow the Irving-Willams series of metal complex stability that identifies an order of metal binding stability (section 1.8.1). Consequently a metal with high binding stability such as copper can dislodge a metal ion with lower stability such as zinc from metalloproteins resulting in their dysfunction.

As previous discussed (section 1.6) metals such as iron and copper can catalyse the production of hydroxyl radicals from hydrogen peroxide. In numerous organisms an

upregulation of reactive oxygen species detoxification systems have been detected upon incubation with high metal concentrations such as copper in *S. cerevisae* or arsenite within *P. aeruginosa* (Parvatiyar *et al.* 2005, Jin *et al.* 2008). Toxic concentrations of metals can damage the integrity of membranes. Copper has been identified to provide a role in lipid peroxidation as previously stated (section 1.6.1, Girotti 1985). Aluminium and Silver have been identified to directly damage membrane integrity (Li *et al.* 2010, Yaganza *et al.* 2004). As previously stated hydroxyl radicals formed by Fenton chemistry can result in DNA damage. Hard lewis acids such as Mn^{2+}, Cr^{4+} and Co^{2+} have been associated with DNA damage but soft lewis acids such as Cu^{2+}, Ni^{2+} and Co^{2+} do not (Nishioka *et al.* 1975). The binding of metals by transport system can inhibit uptake of the intended substrate. Cromate uptake by *S. cerevisae* inhibits the uptake of sulphur and induces a sulphur starvation response (Pereira et al. 2008).

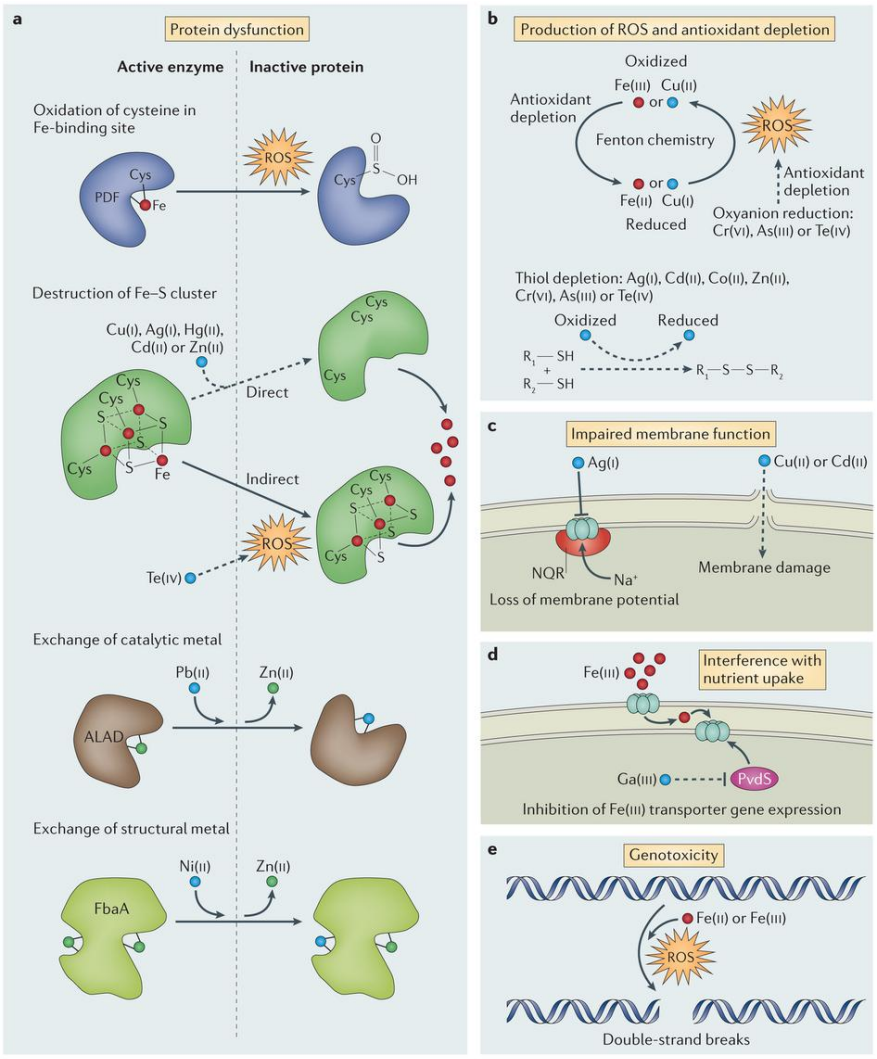


Figure 1.8 Antimicrobial mechanisms of metals

Metals can result in the dysfunction of proteins (a) by competing with other bound metals, in particular iron sulphur clusters. Furthermore, a competing metal can remove a catalytic metal or a metal reuqired structural stabiliy both of which remove functionality of the protein. Redox metals such as copper and iron can catalyse fenton chemistry and the generation of harmful hydroxyl radicals (b). Hydroxyl radicals can damage the cell or can be detoxified by thiol molecules such as glutathione and thredoxin; althought this reduces the thiol pool leaving the cell suceptible to further stresses which thiols detoxify. Metals can bind to transporters inhibiting their action which can lead to membrane damage from a lack of nutrient uptake or over-accumilation of metabolites (c). Additionally metals can inhibit regulators of metal transporters, due to the similarity of metals cross-reactivity is seen within metal response regulators (d). Metals can compriise the integrity of a cell's DNA, it can catalyse Fenton chemistry as previously stated which can result in strand breaks or nucelotide modifications. Taken from Lemire *et al.* 2013.

1.12 Transcriptional regulation of metal homeostasis

1.12.1MerR family regulators

The MerR family of transcription regulators have been identified to respond to diverse molecules including metal ions, small organic molecules and free radicals (Chen and He 2008). The metal sensing MerR family regulators sense a broad range of cations as shown below in table 1.4. The regulators bind to promoters with elongated spacers between the -10 and -35 RNA polymerase binding sites. The -10 and -35 sites typically have a 16-18 bp distances between the sites although the *merPTAD* operon has a 19 bp spacer, repressing transcription (Hobman 2007). MerR regulators contain conserved cysteine residues that form a metal binding domain. The binding of a metal ion induces a MerR regulator dependent allosteric change to DNA at the MerR operon site, unwinding DNA to enable the σ^{70} RNA polymerase to bind the -10 and -35 sites. Without the confirmational change to DNA RNA polymerase is unable to bind the -10 and -35 sites and initiate transcription (Outten *et al.* 1999).

Table 1.4 MerR regulators and divalent cations sensed

Metal ion sensed	**MerR sensor**
Copper	CueR / GolS
Cobalt	CoaR
Zinc	ZntR
Lead	PbrR / ZntR
Gold	CueR / GolS
Silver	CueR / GolS
Cadmium	ZntR
Mercury	MerR
Nickel	NimR

(Brown *et al.* 2003, Kid *et al.* 2011)

The 19 bp spacing between the -10 and -35 RNA binding site is essential to MerR regulation. A decreased spacing (<19 bp) results in the constitutive expression due to RNA polyermase being capable of binding to the -10 and -35 binding sites without MerR-mediated DNA unwinding. Whereas, increased spacing between the -10 and -35 binding sites inhibits expression of the MerR regulated operon as the distance between RNA binding sites is too great for RNA polymerase even after DNA unwinding by MerR regulator activation (Parkhill and Brown 1990).

MerR regulators CueR, MerR and ZntR are capable of a rapid response to the presence of their respective detected metals, activating transcription to aid detoxification before toxic concentrations are reached (Brown *et al.* 2003). MerR regulators are capable of binding and responding to several different metal ions such as CueR binding Cu^{+}, Ag^{+} and Au^{+}, although the binding of other unintentional metals does not result in the activation of the *mer* regulated operon. The metal binding domains of MerR regulators are located within a carboxyl terminus metal binding loop (Osman and Cavet 2010). The metal binding domain can bind multiple metals but has the tightest affinity for the intended metal, otherwise metals will bind to the region following the Irving-Willams metal binding stability series (section 1.8.1). Hence, the binding of a metal by a MerR regulator alone does not activate DNA, MerR regulator activity is based on: amides present metal binding domain determining specificity, allosteric change of regulator and availability of metals (Osman and Cavet 2010). S. Typhimurium CueR has been identified to bind Cu^{+}, Ag^{+} and Au^{+} but only induces the expression of CopA and CueO in response to copper and gold (Stoyanov and Brown 2003). Furthermore, GolS has been identified to bind Cu^{+}, Ag^{+} and Au^{+}, and both gold and copper induces expression of the *gol* system (Checa *et al.* 2007, Osman *et al.* 2010). The binding of Au^{+} by CueR and GolS induces a greater repsonse in both cop and gol systems than Cu^{+} (Checa *et al.* 2007, Osman *et al.* 2010).

1.12.2 Two component copper homeostasis regulation

A typical two component signal transduction systems consist of a sensor kinase and response regulator that work in tandem to regulate gene expression (reviewed Stock *et al.* 2000). The sensor kinase (within Gram negative bacteria) is present within the cytoplasmic membrane and detects the levels of a substrate within the periplasm through two looped domains. Once levels of the substrate reach above or below a threshold value the sensor kinase can bind ATP and transfers a phosphate group from ATP to a conserved histidine residue within its cytoplasmic domain (Krell *et al.* 2010). Phosphorylation of the cytoplasmic

domain of the sensor kinase recruits the response regulator and transfers a phosphate molecule from the histidine residues of the sensor kinase to a conserved aspartate residue on two response regulator monomers. Two response regulators undergo a conformational change upon phosphorylation, dimerise and upregulate gene expression (Stock *et al.* 2000). A sensor kinase can also remove a phosphate group from a phosphorylated response regulator through its phosphatase activity altering gene expression upon an environmental stimulus. For example, in a high Mg^{2+} concentration PhoQ phosphatase activity is induced and removes a phosphate group from PhoP (Castelli *et al.* 2000). A two component signal transduction system in *E. coli* regulates the expression of the plasmid encoded copper detoxification system Pco. Also, *Corynebacterium glutamicum* and *Pseudomonas syringae* both regulate their copper homeostasis systems through a two component system CopRS (Schelder *et al.* 2011, Hernandez-Montes *et al.* 2012). Similarly, the *cus* system within *E. coli* is upregulated by CusRS which is thought to detect periplasmic copper levels (section 1.14, Munson *et al.* 2000). However, examples of modified two component systems are present that include a third regulator that can be activated by the sensor kinase and regulate gene expression of alternate genes to the response regulator. Examples of three regulatory systems are RocS1/RocR/RocA1 in *P. aeruginosa* and BvgS/BvgR/BvgA in *Bordetella pertussis* (Kulasekara *et al.* 2005, Merkel and Stibitz 1995).

1.12.3 CopY copper homeostasis regulation

CopY regulates copper homeostasis within *E. hirae*. CopY regulation is reliant upon a conserved TACAXXTGTA binding sequence within an operon, which CopY binds to (Portmann *et al.* 2004). CopY in low copper conditions forms a dimer containing a Zn^{2+} ion within each monomer that binds to DNA and inhibits transcription of the *copZYAB* operon. In the presence of copper, two Cu^{+} ions displace each zinc ion within each CopY monomer causing a conformational change within CopY and inhibiting its ability to bind to DNA, relieving repression (Cobine *et al.* 1999). The acquisition of copper by CopY is dependent on the copper metallochaperone CopZ supplying the copper to CopY. CopY contains a binding sequence of CXCXXXXCXC which provides the ability to bind either one Zn^{2+} ion or two Cu^{+} ions (Combine *et al.* 1999). Loss of CopY repression triggers expression of the *copYZAB* operon which in addition to CopZ and CopY encodes CopA, which is suggested to import copper from the external environment and CopB that exports excess copper out of the cytosol (Solioz and Stoyanov 2003).

1.13 Mutlicopper oxidases

Multicopper oxidases are enzymes that catalyse a variety of reactions through their redox capabilities due to the presence of copper ions within the enzyme. Multicopper oxidase enzymes have been identified to provide a role in modifying the oxidation states of; copper, billirubin and lactate (Sakurai and Kataoka 2007). Mutlicopper oxidases contain four copper ions; three ions form a trinuclear centre that donate electrons whilst the fourth copper ion is involved in mediating transport of electrons to the substrate (Roberts *et al.* 2002). Multicopper oxidase enzymes catalyse the four electron reduction of oxygen into water and couple this reaction to four electron oxidation reactions (Solomon *et al.* 1996). During catalysis a multicopper oxidase produces oxygen radicals although they are not released from the enzyme, conserved amide residues of aspartic and glutamic acid donate a proton to the radicals which initiates their decay (Bertrand *et al.* 2002).

The multicopper oxidases (CueO) present in *E. coli* and *S. enterica* are part of their *cue* copper homeostasis systems and are located in the periplasm. CueO is transported by the twin arginine pathway and consequently believed to be folded in the cytosol prior to transport. CueO provides a role in copper tolerance for *E. coli* and S. Typhimurium. It is hypothesised that CueO oxidises Cu^{+} to Cu^{2+} in the periplasm which is suggeted to reduce the diffusion of copper into the cytosol (Changela *et al.* 2000). The detoxification of Cu^{+} to Cu^{2+} would also prevent copper-mediated Fenton chemistry in the presence of hydrogen peroxide. CueO within *E. coli* has also been identified to provide an indirect role in copper homeostasis by oxidising the iron siderophore enterobactin which indirectly inhibits its ability to reduce Cu^{2+} (Grass *et al.* 2004). Within *E. coli* a *cueO* mutant has been suggested to contribute to copper homeostasis under anaerobic conditions if the *cus* system is non-functional (Grass and Rensing 2001). Although Tree *et al.* (2005) reported a *cueO* mutant exhibited copper sensitivity in defined media. In the absence of CueO, accumulation of copper occurs within the cytoplasm indicating CueO may indeed have a role in exportation of copper (Tree *et al.* 2005).

The importance of CueO differs between *E. coli* and *S. enterica*. CueO provides a role in copper tolerance under both aerobic and anaerobic conditions for S. Typhimurium, with a *cueO* mutant having reduced growth in the presence of copper to that of wildtype S. Typhimurium (Achard *et al.* 2010). A *cueO* mutant has significantly greater sensitivity to copper under anaerobic conditions than aerobic, although it is currently not known why (Achard *et al.* 2010). Multicopper oxidases provide a role in virulence for several pathogens including; *S. aureus*, *C. jejuni* and S. Typhimurium (Sitthisak *et al.* 2005, Achard *et al.* 2010).

1.14 *E. coli* copper detoxification

E. coli has two defined copper homeostasis systems; *cue* and *cus*. The *cue* system is the primary copper responsive system that regulates the levels of copper within *E. coli*. The *cus* system is only induced when toxic levels of copper are present within *E. coli* under aerobic conditions, whereas under anaerobic conditions the *cus* system is induced under non-toxic intracelluar copper concentrations (Outten *et al.* 2001).

The *cue* system consists of CopA, CueR and CueO. CopA is a copper exporting P_{1B}-type ATPase present within the cytoplasmic membrane that exports copper from the cytosol to the periplasm. An *E. coli copA* mutant has reduced copper tolerance (Rensing *et al.* 2000). CueR is a copper sensing MerR-family transcription factor that detects copper at a zeptamolar concentration (10^{-21}) and upregulates expression of *copA* and *cueO* (Changela *et al.* 2003). The sensitivity of CueR copper detection indicates less than one free copper ion is present within an *E. coli* bacterium. CueO is a multicopper oxidase present in the periplasm that converts Cu^{+} into Cu^{2+} (Outten *et al.* 2001, Grass and Rensing 2001).

The *cus* system belongs to the RND family of exporters and is capable of exporting copper from the cytosol and the periplasm to the external environment. The *cus* system consists of two operons: *cusCFBA* and *cusRS*. CusRS is a two component sensor regulator that detects copper within the periplasm and upregulates *cusCFBA* expression (Munson *et al.* 2000). CusA is a copper ATPase exporter within the cytoplasmic membrane that is suggested to export copper into the periplasm. CusF is a copper chaperone that binds copper within the periplasm and transports copper to CusB, during funnel extraction of copper from the periplasm (section 1.10.4.1, Kim *et al.* 2011). CusB is a membrane fusion protein that links together CusA and CusC. CusC is an outer membrane factor that facilitates the transfer of copper to the external environment. The Cus system provides copper detoxification particularly under anaerobic conditions highlighting its specificity for exporting Cu^{+} and not Cu^{2+}, which is present under aerobic conditions (Outten *et al.* 2001, Beswick *et al.* 1976). *Salmonella* does not possess a *cus* system as *E. coli* and is reliant on alternate copper detoxification methods that are discussed later (section 1.12).

A plasmid encoded copper detoxification systems have also been identified in *E. coli*, Pco (Djoko *et al.* 2008). Pco consists of five genes *pcoACEBD*. PcoA is a multicopper oxidase which can functionally replace a *cueO* mutant or expression of *pcoA* increases copper tolerance although, sequence comparison reveals less similarity than expected (Lee *et al.* 2002). PcoC and PcoE have been identified as copper but do not influence copper tolerance when over expressed in comparison to wildtype *E. coli* (Lee *et al.* 2002, Djoko *et al.* 2008).

The function of PcoB and PcoD remain unknowns, but the mutation of *pcoB* reduces copper tolerance, indicating it is required to achieve maximum copper tolerance from the Pco system (Djoko *et al.* 2008). A PcoRS like two component system that has been identified within *E. coli*, it is hypothesised to upregulate the *cus* system in response to increased levels of copper within the periplasm (Outten *et al.* 2001). PcoRS is capable of activating the expression of CusRS regulated genes in a *cusRS* mutant (Outten *et al.* 2001). S. Typhimurium does not encode a *pco* system although can possess the homologous *silABCRS* system. The *sil* system has been suggested to be involved in the export of silver, with a strain lacking *silABCRS* having significantly reduced silver tolerance (Grupta *et al.* 2001).

Alternatively, *E. coli* has been identified to reduce free copper within the surrounding environment by releasing the iron chelator yersiniabactin, which can also bind copper. *E. coli* does not uptake the yersiniabactin-copper complex reducing the concentration of free copper in the surrounding environment capable of diffusing within *E. coli* (Chaturvedi *et al.* 2012).

Numerous copper detoxification pathways have been identified in several pathogenic organisms including: *E. hirae*, *E. coli*, S. Typhimurium, *L. monocytogenes*, *M. tuberculosis*, *S. cerevisiae* and *C. neoformans* to name a few. Copper detoxification system mechanisms of detecting copper and removing free copper varies between organisms. The presence of a copper detecting transcription regulator that responds to low levels of copper, a P_{1B}-type ATPase that exports copper to either a separate compartment away from the cytosol or external to the bacterium and metallothionein expression to bind copper are all utilised by bacteria.

1.15 The importance of copper homeostasis to *Salmonella* and its virulence

1.15.1 Copper homeostasis in *Salmonella*

The *Salmonella cue* system contains: CopA a P_{1B}-ATPase, CueO a mutlicopper oxidase, CueP a copper binding periplasmic protein and CueR copper binding transcriptional regulator (Espariz *et al.* 2007 Osman *et al.* 2010). The *cue* system is upregulated by copper stress in both aerobic and anaerobic conditions (Osman *et al.* 2010). Although the *cue* system is conserved within *Salmonella* and *E. coli*, *E. coli* does not possess the periplasmic copper binding protein CueP and *Salmonella* does not encode a *cus* system capable of exporting copper outside of its outer membrane. The *cue* system is not the only copper

homeostasis system with S. Typhimurium, the *gol* system also contributes to copper homeostasis. The *gol* system was originally reported to be primarily involved in gold resistance within *Salmonella* but further research has identified its role in copper homeostasis as well (Checa *et al.* 2007). The *gol* system consists of *golSTB*: GolT, a P_{1B}-ATPase, GolB a cytoplasmic protein with sequence similarity to Atx1/CopZ copper chaperones, and GolS, a second CueR-like transcriptional regulator (Checa *et al.* 2007, Osman *et al.* 2010). Checa *et al.* (2007) and Osman *et al.* (2010) reported that GolS provides transcriptional regulation of the *gol* operon in response to an increase in gold and copper levels in the cytosol.

Indeed, a number of redundancies between the *cue* and *gol* systems have been identified supporting the role of the *gol* operon in copper detoxification. Both CueR and GolS can cross regulate the *gol* and *cue* operons respectively, only in a *cueR/golS* double mutant is *Salmonella* unable to upregulate the *cue* and *gol* operons and is hypersensitive to copper toxicity (Osman *et al.* 2010). CueR within *E. coli* was identified to have a zeptamolar affinity for copper (10^{-21}), which is suggested that less than one free atom of cytosolic copper is present in *E. coli*; and is consequently believed CueR within *Salmonella* has a similar affinity (Changela *et al.* 2003). Osman *et al.* (2013) showed that both CueR and GolS have a similar affinity for copper *in vivo*, implying that GolS also is capable of detecting copper at a zeptamolar concentration. However Ibanez *et al.* (2013) identified that although GolS upregulates expression of the *gol* operon in response to copper, GolS has a higher induction in response to Au than Cu. Both CueR and GolS metal binding loops are capable of binding Cu^{+} and the larger ion Au^{+}, the introduction of proline at 113 and 118 residue positions increased and reduced Cu^{+} binding respectively within CueR but does not alter Au^{+} binding (Ibanez *et al.* 2013). Thus identifying residues at positions 113 and 118 within a MerR regulator are important for copper sensing but not gold.

CopA and GolT export copper from the cytosol into the periplasm, removing their ability to catalyse Fenton chemistry or initiate damage within the cytosol. The transport of copper from the cytosol to the periplasm is believed to be important in the removal of copper from *Salmonella*, but it is currently unknown how copper is removed from the periplasm. The export of copper from the cytosol to the periplasm is also suggested to provide a copper supply to periplasmic copper proteins such as CueO and CueP. CopA and GolT are required to metallate the copper chaperone CueP, A *copA/golT* double mutant could not metallate CueP, and consequently CueP could not supply $SodC_{II}$ with copper (Osman *et al.* 2013). A single mutant for *golT* does not influence copper tolerance, whereas a *copA* mutant has reduced copper tolerance than wildtype but not a significant difference. Single mutants for

copA and *golT* also do not exhibit any difference in copper accumulation to wildtype. Also, a *copA* or *golT* single mutant do not exhibit sensitivity to growth within cell cultured macrophages (Osman *et al.* 2010). Both P_{1B}-type ATPases are capable of functionally replacing the other in a single mutant and only when both exporters are knocked out *Salmonella* has significantly reduced survival in the presence of copper. A *copA/golT double* mutant has significantly reduced copper tolerance, over-accumulates copper at sub-lethal copper concentrations (Osman *et al.* 2010).

CueO catalyses the oxidation of Cu^{+} to Cu^{2+} in the periplasm of both *Salmonella* and *E. coli* (section 1.13). It is suggested that Cu^{2+} has reduced ability to diffuse into the cytosol than Cu^{+} due to possessing an increased charge than Cu^{+} (Grass and Rensing 2001). A *Salmonella cueO* mutant has reduced copper tolerance under both aerobic and anaerobic conditions; with substantially reduced survival under anaerobic conditions (Achard *et al.* 2010). *E. coli* contain the *cus* copper efflux system that has been identified to be active under anaerobic conditions which *Salmonella* does not possess, which could explain the increased importance of CueO under anaerobic conditions within *Salmonella*.

Copper profiling experiments identified the presence of a copper complex in the periplasm of *Salmonella* and this protein was identified as CueP (Pontel *et al.* 2009, Osman *et al.* 2010). A *cueP* mutant does not contribute to copper tolerance under aerobic conditions but has been suggested to provide a role in copper tolerance under anaerobic conditions (Pontel *et al.* 2009). The mechanism of CueP copper resistance is not fully understood but its regulation is under control of CueR. CueP has recently been identified to perform a chaperone function supplying copper to Cu,Zn $SodC_{II}$, $SodC_{II}$ within a *cueP* mutant cannot acquire copper and consequently is unable to detoxify superoxide (Osman *et al.* 2013). An overview of *S. typhiumirum* copper homeostasis is shown in figure 1.8

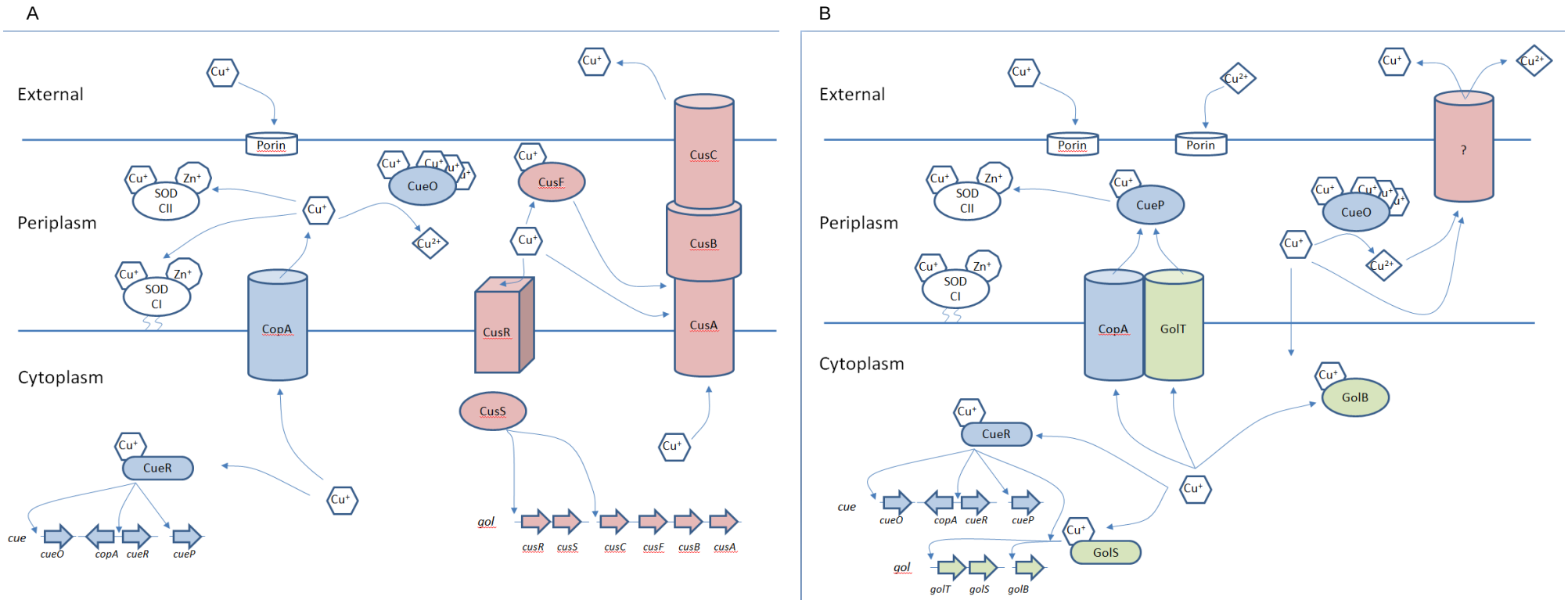


Figure 1.9 Overview of copper homeostasis within *E. coli* and S. Typhimurium

Copper is thought to enter both *E. coli* and *Salmonella* through porins present in the outer membrane by diffusion, and Cu^{+} is suggested to diffuses into the cytosol. Cu^{+} is detected within *E. coli* (A) by CueR that induces expression of the *cue* operon, CusS within the periplasm activates CusR which then induces expression of the *cus* system. CopA exports Cu^{+} from the cytosol into the periplasm, where CueO converts Cu^{+} into Cu^{2+}. Cu^{+} is also supplied to $SodC_{I}$ and $SodC_{II}$ but is currently unknown how. CusABC exports copper outside of *E. coli*, it is unclear if Cu^{+} is exported from the cytosol or the periplasm; and whether CusF is required for binding and transfer of copper to CusA. Cu^{+} is detected by CueR and GolS in *Salmonella* (B) which induce expression of *cue* and *gol* regulons respectively, although cross regulation can occur. The role of GolB in copper homeostasis remains unknown but possess sequence similarity to known copper chaperone Atx1/CopZ. CopA and GolT both export Cu^{+} from the cytosol into the periplasm, providing functional redundancy. CueP is a metallochaperone that receives copper from CopA and GolT and supplies copper to $SodC_{II}$. CueO converts Cu^{+} into Cu^{2+} within the periplasm. Currently there is no known outer membrane copper exporter.

1.15.2 *Salmonella* copper homeostasis during intracellular survival

Previous work has indentified that a *copA/golT double* mutant has decreased survival during competitive infections of cell cultured macrophages against wildtype S. Typhimurium (Osman *et al.* 2010). Indeed, from 12 hours post infection a drop in a *copA/golT double* mutant replication is seen along with an increase in expression of *copA* and *golT* (Osman *et al.* 2010). This confirmed that S. Typhimurium is exposed to copper during intracellular survival within macrophages. Infections of S. Typhimurium within mice identified a *cueO* mutant had reduced survival in comparison to its parental strain. Although, *in vitro* assays with macrophage cell line Raw 264.7 or bone marrow derived primary macrophages exhibited no difference in survival (Achard *et al.* 2010). This indicated CueO provides a role in survival within a mouse model that *in vitro* infection assays do not replicate such as cytokines produced in response to S. Typhimurium infection.

White *et al.* (2009) identified that expression of ATP-7a increases upon infection of *E. coli* within cell culture macrophages, and that ATP-7a associates with the phagosome. Achard *et al.* (2012) also reported an increase in both expression and abundance of ATP-7a when S. Typhimurium infected macrophages, although unlike *E. coli*, ATP-7a did not associate with the phagosome. It is possible that virulence factors within SPI-2 inhibit the association of ATP-7a with the SCV as occurs with iNOS and NADPH oxidase (Vazquez-Torres *et al.* 2000). The mechanism by which copper provides antimicrobial killing within macrophages remains open. Whether copper is directly pumped into the SCV or released from complexes with S. Typhimurium is unknown.

1.16 Aims of the project

It is known that copper has significant antimicrobial properties and hence, in addition to acquiring copper for cuproprotein function S. Typhimurium must also avoid copper-mediated toxicity. Recent findings have indicated a role for copper in the bactericidal activity of macrophages, and the ability of S. *typhimurium* to export copper, via either one of its two related copper-resistance associated P_{1B}-type ATPases, CopA and GolT, aids survival in macrophage phagosomes. However, the precise mechanism of copper-toxicity within macrophages remains unknown, although speculations include a role for copper in respiratory burst oxidase mediated killing, due to its participation in Fenton chemistry, and/or in potentiating nitrosative stress. This study therefore aimed to investigate the role of the S. Typhimurium copper homeostatic systems in providing resistance to reactive oxygen and nitrogen species, in the presence and absence of copper, and the contributions of these species to the bactericidal affect of copper in macrophages. In addition, although copper-resistance in S. Typhimurium is associated with two characterised copper homeostatic systems, *cue* and *gol*, that function to reduce the cellular copper load, it is not known how copper is exported across the outer-membrane. A further aim of this study was therefore to identify a transporter involved in the export of copper across the S. Typhimurium outer membrane.

The specific aims of this study were:

1. To determine whether or not copper influences the antimicrobial potency of reactive oxygen and nitrogen species against S. Typhimurium and the role of the known S. Typhimurium copper homeostatic proteins in protecting against these species.

2. To examine the role of the S. Typhimurium copper homeostatic proteins during infection of macrophages, and the contribution of reactive nitrogen species to the bactericidal affect of copper in these cells.

3. To investigate the role of TolC in copper-export across the outer-membrane of S. Typhimurium

2 Materials and Methods

2.1 Bacterial strains, storage and growth conditions

S. Typhimurium and *E. coli* strains used during this study are shown in table 2.1, with S. *typhimurium* SL1344, a histidine auxotroph, being used as a representative wildtype strain in this study. Strains were stored long term at -80°C in 50% (v/v) glycerol. For short term use, strains were streaked from -80 °C stocks onto solid media plates and stored at 4°C for up to three weeks. Aerobic cultures were grown at 37°C with shaking at 200 revolutions per minute (rpm); anaerobic cultures were grown at 37°C statically in syringes. Bacteria were grown using either rich Luria-Bertani (LB) media or defined minimal (M9) media supplemented with 20 µg ml^{-1} histidine (M9H). LB media contained; 10 g l^{-1} NaCl, 10 g l^{-1} tryptone, 5 g l^{-1} yeast extract in milliQ water (mQH_2O). Minimal media consisted of 1x M9 salts, 0.4% (v/v) glucose, 2 mM $MgSO_4$, 100 µM $CaCl_2$ in sterile mQH_2O. 5x M9 salts consisted of; 35.9 g l^{-1} Na_2HPO_4, 15 g l^{-1} KH_2PO_4, 2.5 g l^{-1} NaCl and 2.5 g l^{-1} NH_4Cl. Glassware used for minimal media or minimal media components (added to give required final concentrations above) was acid washed in 4% nitric acid overnight to remove metal ions from the surface and then washed thoroughly with milliQ H_2O prior to use. Solid media was prepared by adding 15 g l^{-1} agar prior to autoclaving after which media was poured into sterile Petri dishes (SLS) while still molten and left to cool and solidify. Bacteria with antibiotic resistance cassettes were selected by addition of chloramphenicol 10 or 34 µg ml^{-1}, kanamycin 50 µg ml^{-1} or ampicillin 100 µg ml^{-1}. Optical densities were measured at 600 nm using either an Ultrospec 2100 pro (Amersham Biosciences) spectrophotometer or a Synergy HT (Bio-Tek) plate reader. For measurement in the spectrophotometer 1 ml of culture was transferred into a 2 ml plastic cuvette (Fisher). For measurement by plate reader, 300 µl of culture was transferred into a single well of a 96 well plate (Fisher).

Table 2.1 Strains used within study

Strain	Genotype	Reference / source
	E. coli	
DH5α	*F⁻ Φ80 lacZ*ΔM15 *Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK–, mK+) phoA* sup*E44 λ–* thi-*1* gyr*A96*	EGSC

	*rel*A1	
S. Typhimurium		
LB5010a	*metA22 metE551 ilv-452 leu-3121 trpΔ2 xyl-404 galE856 hsdLT6 hsdSA29 hsdSB121 rpsL120*	SGSC
LB5010a *ΔgesBC*	LB5010a *ΔgesBC.: cat*	This study
LB5010a *ΔkatG*	LB5010a *ΔkatG.: cat*	This study
LB5010a *ΔtolC*	LB5010a *ΔtolC.: cat*	This study
SL1344	*hisG46*	SGSC
SL1344 *ΔcopA*	SL1344 *ΔcopA.: scar*	Osman *et al.* 2010
SL1344 *ΔcopA cat*	SL1344 *ΔcopA.: cat*	Osman *et al.* 2010
SL1344 *ΔcopA / ΔgolT*	SL1344 *ΔcopA / ΔgolT.: scar*	Osman *et al.* 2010
SL1344 *ΔcopA / ΔgolT* cat	SL1344 *ΔcopA / ΔgolT.: cat*	Osman *et al.* 2010
SL1344 *ΔcopA / ΔgolT / ΔsodC$_I$ / ΔsodC$_{II}$ cat*	SL1344 *ΔcopA / ΔgolT / ΔsodC$_I$ / ΔsodC$_{II}$.: cat*	Osman *et al.* 2013
SL1344 *ΔcueO*	SL1344 *ΔcueO.: scar*	Osman *et al.* 2010
SL1344 *ΔcueO / ΔcueP*	SL1344 *ΔcueO / ΔcueP.: scar*	This study
SL1344 *ΔcueP cat*	SL1344 *ΔcueP.: cat*	This study
SL1344 *ΔcueP*	SL1344 *ΔcueP.: scar*	Osman *et al.* 2010
SL1344 *ΔgesB / ΔgesC*	SL1344 *ΔgesB / ΔgesC.: scar*	This study
SL1344 *ΔgolB*	SL1344 *ΔgolB.: scar*	Osman *et al.* 2010

SL1344 *ΔgolT cat*	SL1344 *ΔgolT.: cat*	Osman *et al.* 2010
SL1344 *ΔgolT*	SL1344 *ΔgolT.: scar*	Osman *et al.* 2010
SL1344 *Δhmp*	SL1344 *Δhmp.: kan*	Crawford and Goldberg 1998b
SL1344 *Δhmp / ΔcopA / ΔgolT*	SL1344 *Δhmp / ΔcopA / ΔgolT.: kan*	This study
SL1344 *ΔkatG*	SL1344 *ΔkatG.: scar*	This study
SL1344 *ΔkatG / $\Delta sodC_I$ / $\Delta sodC_{II}$*	SL1344 *ΔkatG / $\Delta sodC_I$ / $\Delta sodC_{II}$.: scar*	This study
SL1344 *ΔkatG / ΔcopA / ΔgolT*	SL1344 *ΔkatG / ΔcopA / ΔgolT.: scar*	This study
SL1344 *$\Delta sodC_I$ / $\Delta sodC_{II}$*	SL1344 *$\Delta sodC_I$ / $\Delta sodC_{II}$.: scar*	Osman *et al.* 2010
SL1344 *$\Delta sodC_I$ / $\Delta sodC_{II}$ cat*	SL1344 *$\Delta sodC_I$ / $\Delta sodC_{II}$ cat.: cat*	This study
SL1344 *ΔtolC*	SL1344 *ΔtolC.: scar*	This study
SL1344 *ΔtolC / ΔcueP*	SL1344 *ΔtolC / ΔcueP.: scar*	This study
SL1344 *ΔtolC / ΔcueO*	SL1344 *ΔtolC / ΔcueO.: scar*	This study

2.2 Chemical reagents

Chemicals were purchased from Sigma unless stated otherwise. When required chemicals were sterilised by either autoclaving (121°C and 15 psi for 20 min) or by filter sterilisation using 0.22 µm filters (Millipore).

2.3 DNA Manipulation

2.3.1 Polymerase chain reaction (PCR)

PCR was set up using; >1 ng DNA, 1 µM 5' primer, 1 µM 3' primer, 250 µM dNTP's (Biorad), 1 x Taq buffer, 1 unit Taq (Roche) and mQH_2O to a volume of 50 µl. For synthesis of products > 2 kbp or use of primers > 40 nucleotides (nt) in length, 1 unit Pwo (Roche) was included in the PCR mix for its proof-reading capabilities. For colony PCR an individual colony was stabbed into a microtube containing PCR reagents for the source of DNA. PCR was performed using a Uvigene thermal cycler. Primers were obtained from MWG Eurofins and re-suspended in mQH_2O according to supplier's synthesis report. A full list of primers used in this study is given in table 2.2.

Typical PCR cycle;

- Hot start 95°C 30 sec
- Denaturing 95°C 30 sec
- Annealing 45-55°C 30 sec*[1]
- Extension 72°C 1-4 mins*[2]

} 30 cycles

- Final extension 72°C 5 mins

*[1] Annealing temperature was changed specific to primer synthesis report recommendation.
*[2] Elongation time was adjusted to enable complete synthesis of the gene using a guide rate of 1 kb min^{-1}.

Table 2.2 Primers used within project

Primer	**Primer sequence 5' - 3'**
copA forward	GAC CTT AAC CTT GCT GGA AGG
copA_RT forward	CGA CCT GAC CCT GGA CGG TTT GTC C
copA reverse	GCT GAT GCT GCC TGA TAT AGC
copA_RT reverse	CGC AGT GCG TTC CGC CAG GTT TAC C

cueP forward	GCA TTA CTT TAC CCT GCG TCC
cueP reverse	GGT CAG TAC AAA GTG AGC CCA C
cueO forward	AGG ATT GGT CGC GGC GTT TTC
cueO reverse	CGT TTG GTA CGA AGA TGG GCG
gesB_del forward	ACA TTG ACT AAA GCG GAC GGC GAC AGT GCG CCG AAG GCG GTG CGC CAA TGT GTG TAG GCT GGA GCT GC
gesB forward	GGT GCG TCC TGG CAT GAC CG
gesB_del reverse	AAG TGA TCT TCA TTA TGC CAT TCC CAT CAT TAT GCT TGC TGA TCA TGC GAC ATA TGA ATA ACT CCT CT
gesB reverse	GCC AAT GTC AGC GGC ATC CG
gesC_del forward	CCG CGT TGA TTC GCA TGA TCA GCA AGC ATA ATG ATG GGA ATG GCA TAA TGT GTG TAG GCT GGA GCT GC
gesC_del reverse	CCG CAC TGG CGA CGC GGG CGC TAT TTG GGC TAA CCT TGC TTT TTC TCA CTC ATA TGA ATA TCC TCC CT
gesC forward	GCT CTT CGG CCT GTT ATT GAC G
gesC reverse	CCT GTT AAC CCA GCG TGC AGG C
golB forward	CAT ACG CTT GGA CAA CCT GAC
golB reverse	GGG GAT TAT CTC ACG CAA AG
golT forward	GCA AAG GCC CAG AAC AGA TTC
golT_RT forward	GGT ATG ACC TGC GCG TCG TGC GTC G
golT reverse	GTG GCG TAA ATG TCT CGC ATC
golT_RT reverse	GGC GAC CAG CGA GTT CAT GTC CGG C
hmp forward	GCT GGT TGA AAC AGG ACC

hmp reverse	CCG CAA AGA TAG AAC TGC
katG_del forward	GCT CCT GGT GTA TAT CGT AAC GGT AAC ACT TTA AAA GGG AGC TGA GAT ATG GTG TAG GCT GGA GCT GCT TCG
katG forward	CCA CAC GCT GGG CGT AAG ACC
katG_del reverse	TAG CAG CCG CTG ACG AAT TAA CCT GTC AGA TTA TTG CAG ATC GAA ACG GTC CAT ATG AAT ATC CTC CTT AG
katG reverse	GGC ACC CCT TCA GGC GTG ACG
rpoD_RT forward	GCT GAA ACT TCT TGT CAC CCG TGG
rpoD_RT reverse	CGG AGC ATT GAA CCT GGT TGA TCC
sodC$_I$ forward	GCC AAT CGC GGT TAA ATC
sodC$_I$ reverse	GGC GTA GGT TAC AGC TTC
sodC$_{II}$ forward	GTG CCG TAA TCG CCA ACT CG
sodC$_{II}$ reverse	GGC GAT TAT CTG GCG TTT ACA C
tolC forward	GGA TTC TGC TAG AAT CAG C
*tolC*_comp forward	AAT GAT GGA TCC TAG AAT TTC AAG GGT GGT TGA CTG
*tolC*_del forward	GCT CCC CAT CCT TAT CGG CCT GAG CCT GTC GGG GTT CAG CGT GTA GGC TGG AGC TGC TTC
tolC reverse	GGT CTG ATA AAC GCA GCG C
*tolC*_comp reverse	GCC TTC AAG CTT GAG GAT GAC TGG TCG AAA TTG
*tolC*_del reverse	GCC GGA ATG GAT TGC CGT TAT TGC TGT TGG CGC GAG CGG CCA TAT GAA TAT CCT CCT TAG
16s_RT	GCC ATG CCG CGT GTA TGA AGA AGG

forward	
16s_RT reverse	CCA CGC TTT CGC ACC TGA GCG TCA

2.3.2 PCR product purification

PCR product purification was performed with a QiaQuick PCR purification kit (Qiagen) according to manufacturer's instructions. Products were analysed by agarose gel electrophoresis and quantified on a Nanodrop ND-1000 UV spectrophotometer (Labtech) using the 260-280 nm absorption value.

2.3.3 Extraction of plasmid DNA

A full list of plasmids used in this study is given in table 2.3. Overnight bacterial cultures (5 ml and 10 ml for high and low copy number plasmids, respectively) were pelleted by centrifugation at 3,000 g for 20 mins (Fisher Scientific Accuspin 1R), the supernatant discarded, and plasmids purified by alkaline lysis using the Qiagen plasmid purification kit, according to manufacturer's instructions. For extraction of plasmids from S. Typhimurium strains the protocol was modified slightly, such that following addition of neutralisation buffer, tubes were inverted twenty times and the sample pelleted by centrifugation at 14,000 g for 20 mins, followed by plasmid DNA being eluted with 50 µl nuclease free mQH_2O. Plasmid DNA was analysed on a Nanodrop or by agarose gel electrophoresis. Plasmids were typically propagated in *E. coli* DH5α and introduced into the restriction-deficient modification-proficient S. Typhimurium strain LB5010a prior to transfer to wildtype, SL1344.

Table 2.3 Plasmids used within project

Plasmid	**Description**	**Reference/source**
paCYC184	Cloning vector, Chl^r	Chang and Cohen 1978
paCYC184 *tolC*	Cloning vector encoding *tolC*, Chl^r	This study

pCP20	Cloning vector containing flip recombinase enzyme, Amp^r	Cherepanov and Wackernagel 1995
pKD3	Cloning vector containing chloramphenicol resistance cassette flanked by flip recombinase sites, Chl^r	Datsenko and Wanner 2000
pKD46	Cloning vector containing lamba red recombinase system	Datsenko and Wanner 2000
pRS451 *copA*	Transcriptional fusion *lacZ* reporter plasmid encoding P*copA* from -127 to +29, Amp^r	Osman *et al.* 2010
pRS451 *golT*	Transcriptional fusion *lacZ* reporter plasmid encoding P*golT* from -286 to +9, Amp^r	Osman *et al.* 2010

2.3.4 Extraction of genomic DNA

Overnight bacterial cultures were pelleted by centrifugation at 3,000 g for 20 mins. The supernatant was discarded and genomic DNA purified by alkaline lysis using the Gentra Puregene cell kit (Qiagen), according to manufacturer's instructions. Genomic DNA was analysed on a Nanodrop or by agarose gel electrophoresis.

2.3.5 DNA sequencing

All plasmid constructs used within this study were checked by DNA sequencing. DNA sequencing reactions were carried out using the Big Dye V1.1 Terminator Cycle Sequencing Kit (Applied Biosystems) according to manufacturer's instructions. Reactions contained 500-750 ng of DNA, 0.1 µM primer, 3 µl Big Dye sequencing buffer, 2 µl Big Dye terminator mix and topped up to a 20 µl volume with nuclease free mQH_2O. The PCR sequencing cycle used was:

- 95°C 2 mins
- 95°C 40 sec
- 50°C 15 sec
- 60°C 4 sec

} 25 cycles

PCR samples were purified by ethanol precipitation. Ethanol precipitation involved: addition of 60 µl 100% (v/v) ethanol and 5 µl 125 mM EDTA followed by incubation at room temperature for 15 mins and pelleted by centrifugation at 14,000 g for 30 mins and removal of the supernatant. The pellet was then washed by re-suspension in 60 µl 70% ethanol (v/v) and incubated at 37°C for 5 mins, followed by being pelleted by centrifugation at 14,000 g for 5 mins and supernatant removed. DNA sequencing was subsequently performed by Paul Fuller (at the DNA Sequencing Facility, University of Manchester) and results were checked against known DNA sequence, with chromatograms analysed with Cromas 233.

2.3.6 Plasmid digestion

1 µg of purified plasmid was incubated with 1 U of each desired restriction enzyme (Roche) with a mutually functional buffer as indicated by supplier's instructions and incubated at 37°C for 2 hrs. The resultant DNA was screened by agarose gel electrophoresis to ensure digestion was successful.

2.3.7 Plasmid ligation

A ligation mix consisting of; 1 ug insertion DNA and 0.25 ug vector DNA was mixed together with 1 U DNA ligase (Roche) and 1x buffer B and incubated at room temperature for 4 hrs. DNA ligation was screened by agarose gel electrophoresis to ensure the ligation had worked.

2.3.8 Agarose gel electrophoresis

Agarose gel electrophoresis was performed using between 0.5-1.5% (w/v) of agarose in TAE buffer (0.5 M Tris, 5.7% acetic acid, 10 mM EDTA pH 8) essentially as described by Sambrook *et al* (2001). In brief, agarose was melted by heating within a microwave for 1 min on full power and poured onto an EPS 200 gel chamber (Pharma Biotech); then left to

solidify with a comb placed within. Electrophoresis was performed in a gel tank containing TAE buffer supplemented with 1 µg ml^{-1} ethidium bromide. Samples were prepared for gel electrophoresis by adding 6x loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol and 30% (v/v) glycerol) at a 1:5 dilution with the sample. Samples were electropheresed in parallel with a molecular marker of known DNA sizes (Bioline) at 100 V for 40-90 mins then analysed under a UV light transilluminator (UVITec UVIpro silver).

2.3.9 Creating electrocompetent S. Typhimurium cells

An overnight culture of S. Typhimurium was diluted 1/100 into fresh LB broth and grown at 37°C with shaking at 200 rpm until an OD_{600} of 0.2-0.3 on a plate reader was reached. Cells were incubated on ice for 10 mins then pelleted by centrifugation at 3,000 g, 4°C (pre-cooled) for 20 mins. Supernatant was discarded and pellet re-suspended in 10 ml ice cold sterile mQH_2O. Centrifugation and re-suspension were repeated three further times with the third re-suspension with 10 ml ice cold sterile 10% (v/v) glycerol. The cells were pelleted by centrifugation again and re-suspended in 1/40 of initial culture volume in 10 ml ice cold sterile 10% (v/v) glycerol. Competent cell culture was divided into 50 µl aliquot samples and snap-frozen in liquid nitrogen and stored at -80°C.

2.3.10 Transformation of electrocompetent S. Typhimurium cells

A 50 µl aliquot of electrocompetent cells was thawed on ice and then incubated with 1 µg plasmid for 10 mins on ice. The plasmid and electrocompetent cells were then transferred to an electroporation cuvette (Biorad) which had been pre-cooled in ice and electroporation performed at 2.5 kV, 200 Ω and 25 µF. Immediately after electroporation, 1 ml of LB broth was added and cells were allowed to recover by incubation at 37°C with shaking at 200 rpm for 1-3 hrs. Successful transformants were then screened by spread plating 200 µl onto an LB agar plate containing an appropriate antibiotic to select for an introduced antibiotic resistance gene, and grown at 37°C overnight. A candidate colony for a successful transformant was either analysed by colony PCR or grown overnight in LB media and plasmid purified from the overnight culture and digested to confirm the introduced plasmid was correct.

2.3.11 Real-time PCR of S. Typhimurium

2.3.11.1 RNA extraction

An overnight culture was diluted 1/100 into fresh LB broth and grown to an OD_{600} 0.2–0.3 measured on a plate reader then pelleted by centrifugation at 3,000 g for 20 min, and the supernatant discarded. RNA extraction was performed on the pellet using Trizol Pure Link Mini kit following supplier's instructions (Qiagen) with the exception that following annealing of RNA (in 70% (v/v) ethanol) to the column , 1U of DNase 1 and 1x buffer (New England Biolabs) were added to the column and incubated at room temperature for 10 mins. RNA was subsequently eluted in 50 µl of DEPEC treated mQH_2O (treated with 0.1% (v/v) DEPEC and incubated at 37°C with shaking at 50 rpm for 24 hrs followed by autoclaving). RNA quality was checked by diluting 1/2 with 10 mM Tris pH 7.5 and absorbance measured at 260 nm with a Nanodrop. RNA quantity was calculated using the equation:

RNA (µg) = OD_{260} x 40 µg / (OD_{260} x 1 ml) x dilution factor x total sample volume (ml)

RNA was snap-frozen in liquid nitrogen and stored at -80°C until required.

2.3.11.2 Synthesis of cDNA

RNA was thawed on ice and 5 ng transferred to an RNase free microtube in preparation for reverse transcription. The reverse transcription reaction contained; 5 ng RNA, 0.6 µM 3' primer, 250 µM dNTP's, 1 unit reverse transcriptase (Qiagen), 1x reverse transcriptase buffer and DEPEC treated mQH_2O to a volume of 30 µl. Reverse transcription was performed in a PCR unicycler using conditions listed below.

Reverse transcription cycle;

- Reverse transcription 50°C 30 mins
- Denaturing 95°C 30 sec

cDNA formed from reverse transcription was quantified by measuring absorbance at 260 nm on a Nanodrop. Following synthesis cDNA was snap-frozen in liquid nitrogen and stored at -80°C until required for real-time PCR.

2.3.11.3 Real time PCR

A real-time PCR master mix consisted of; 4 µg cDNA, 0.9 µM 5' primer, 0.9 µM 3' primer, 1 unit of SYBR green supermix (Qiagen) and mQH_2O to a volume of 20 µl. A real-time PCR cycle as shown below was used;

- Denaturing 95°C 15 sec
- Annealing 50°C 15 sec
- Extension 72°C 30 sec
- Read plate

30 cycles

- Melting curve 50-92°C, 0.5°C per increase, 2 sec hold

Gene fold expression change was calculated using $2^{-\Delta\Delta CT}$ formula (Winer *et al.* 1999, Kivak 1997). Gene expression was monitored for *16s* rRNA, *rpoD*, *copA* and *golT* using primers denoted as gene name_RT forward and gene name_RT reverse in table 2.2.

2.4 Generation of gene disruption mutants of S. Typhimurium

2.4.1 Gene replacement by an antibiotic resistance cassette

One step PCR mutagenesis was performed according to the protocol of Datsenko and Wanner (2000) and using S. Typhimurium LB5010a that lacks chromosomal DNA restriction systems (Bullas *et al.* 1983). Primers specific to the template plasmid pKD3 were designed to include a 50 nt 5' overhang homologous region to the 5' or 3' region of the gene targeted for removal in addition to the 18 nt region homologous to pKD3. Standard PCR conditions were used with the inclusion of equal amounts (1U) of Taq polymerase and Pwo DNA polymerase (Roche), due to its 3'-5' exonuclease proof-reading activity, to amplify the chloramphenicol resistance cassette of pKD3. Five separate PCR reactions were combined and purified with a QiaQuick PCR purification kit following supplier's instructions (Qiagen). An overnight culture of LB5010a containing plasmid pkD46 was diluted 1/50 in fresh LB broth and grown for 1 hr at 37°C with shaking at 200 rpm prior to the addition of 1 ml of 10 mM L-arabinose and growth continued until early log phase (OD_{600} 0.2–0.3, in a plate reader) was reached. The cells were then made electrocompetent and the purified PCR product (containing homologous target gene regions flanking a chloramphenicol resistance cassette, CAT) was introduced by electroporation. Cells were allowed to recover at 37°C with shaking at 200 rpm for 3 hrs and then plated out onto LB agar plates containing 10 µg ml^{-1} of chloramphenicol and grown at 37°C overnight. Resulting colonies were screened by colony PCR using primers that flank the gene targeted for mutation and products analysed

by agarose gel electrophoresis. If the mutation was successfully created, the mutation was transferred from LB5010a into SL1344 by P22 phage transduction.

2.4.2 P22 phage preparation and transduction

2.4.2.1 P22 phage quantification

Labs stocks of P22 bacteriophage were quantified using LB5010a. Separately 100 µl of phage was diluted in 0.9% (v/v) NaCl and dilutions 10^{-5}–10^{-9} were mixed gently with 100 µl of a LB5010a overnight culture and 4 ml of molten soft agar (0.75% agar) before being poured on top of an LB agar plate. The plates were then incubated at 37°C overnight and plaques present were counted and plaque forming units' ml^{-1} calculated. A plate with confluent plaques from quantifying plaque forming units was selected and 5 ml of 0.9% (v/v) NaCl was poured onto a plate and left overnight at room temperature. Phage soak out was harvested in a 15 ml Falcon tube, giving a phage stock. The phage stock had its plaque forming units determined as previously described and 200 µl chloroform was added to the falcon tube to kill any residual bacteria.

2.4.2.2 Preparation of P22 lysate

Phage stock was diluted to 10^8 pfu in 200 µl 0.9% (v/v) NaCl and mixed with 100 µl overnight culture of donor strain and 4.7 ml of transducing broth then incubated overnight at 37°C with shaking at 200 rpm. Transducing broth consisted of: 48.8 ml LB broth, 1 ml 50x E salts (Vogel and Bonner 1956), 200 µl 50% (v/v) glucose. Controls that contained no donor bacteria and no phage dilution were also performed. Lysates had 100 µl of chloroform added and incubated at 37°C with shaking at 200 rpm for 5 mins then pelleted by centrifugation at 3,000 g for 20 mins. The supernatant was transferred to a fresh tube and 100 µl of chloroform added to give the mutant donor lysate stock.

2.4.2.3 Transduction

Transducing broth was diluted to 10^{-4} in 0.9% (v/v) NaCl, 100 µl of all dilutions (10^0–10^{-4}) were separately mixed with 100 µl of recipient overnight culture and incubated for 1 hr at 37°C with shaking at 200 rpm. After incubation the culture was spread plated onto LB agar plates containing either 10 µg ul^{-1} chloramphenicol or 25 µg ul^{-1} kanamycin depending on

antibiotic resistance cassette and grown overnight at 37°C. Colonies present on antibiotic containing plates were screened by colony PCR to identify if the mutation was correct.

2.4.3 Removal of gene disruption antibiotic cassette

Antibiotic resistance cassettes were removed by the flip recombinase enzyme encoded within pCP20 (Cherepanov and Wackernagel 1995). A strain containing a gene disruption antibiotic cassette was made electrocompetent and pCP20 was introduced by electroporation. Following recovery, 200 µl of culture was spread plated onto LB agar plates containing 100 µg ml^{-1} ampicillin which selected for bacteria containing pCP20, and were incubated overnight at 30°C. A colony was selected from the ampicillin containing LB agar plates and inoculated into 5 ml LB broth overnight and grown at 37°C with shaking at 200 rpm to remove the heat-sensitive plasmid. The overnight culture was serially diluted in PBS to 10^{-9}, four 20 µl droplets of dilutions 10^{-6}–10^{-9} were plated out onto LB agar plates by Miles and Misra technique to obtain individual colonies (Miles and Misra 1938). Fifty colonies from the dilution plates were used to patch onto LB agar plates containing: no antibiotic, 100 µg ml^{-1} ampicillin and 34 µg ml^{-1} chloramphenicol. Colonies that had lost chloramphenicol and ampicillin resistance were checked by colony PCR to confirm the cassette was removed.

2.5 Reactive species killing and tolerance assays

2.5.1 Reactive oxygen species killing assay

Overnight cultures were diluted 1/10 and 30 µl were added to the wells of a 96 well plate containing 200 µl of LB broth, 30 µl 1 mM $CuSO_4$ or H_2O and various concentrations of reactive oxygen species (diluted in H_20) in a volume of 40 µl (added last). The sources of reactive oxygen species used were hydrogen peroxide or paraquat. Plates were incubated at 37°C for 2 hrs. Cultures were subsequently serial diluted in PBS and dilutions plated onto LB agar plates and colonies quantified by Miles and Misra technique.

2.5.2 Xanthine oxidase killing assay

Overnight cultures were diluted 1/100 into 2.445 ml of PBS within a 14 ml tube, with 50 µl 25 mM hypoxanthine or H_2O with and without 5 µl of 50 U bovine xanthine oxidase were added as indicated in individual experiments. Tubes were incubated at 37°C with shaking at 200

rpm for 2 hrs. Cultures were subsequently serially diluted in PBS and dilutions plated onto LB agar plates and colonies quantified by Miles and Misra technique. To confirm the production of superoxide, 25 µM of nitro blue tetrazolium was added and the formation of nitro blue tetrazolium formazan was measured at an absorbance of 560 nm.

2.5.3 Reactive nitrogen species tolerance assay

Overnight cultures were diluted 1/100 into fresh LB broth and 30 µl added to 200 µl of LB broth and 70 µl of reactive nitrogen species and sterile H_2O, depending on the concentration required, in a well of a 96 well plate. A variety of RNS generators were used: acidified sodium nitrite (ASN), GSNO and NOC5/7. Acidified sodium nitrite required sodium nitrite dissolved in H_2O to be mixed with acidified 1M MES (buffered to a pH of 5.5 by drop-wise addition of 1M NaOH) at a 1:1 ratio before addition to the 96 well plate in a fume hood. GSNO was dissolved in mQH_2O and NOC-5 and NOC-7 were dissolved in 0.1% (v/v) NaOH. Cultures were incubated at 37°C for 7 hrs then serially diluted in PBS; with viable bacteria subsequently quantified using Miles and Misra technique plating onto LB agar plates and grown at 37°C overnight. Nitrite levels in media were quantified by the addition of 150 µl of bacterial culture to 150 µl Griess reagent (Calbiochem) and absorbance at 540 nm was measured by a plate reader.

Subsequent experiments examined the effects on growth of selected (from the previous experiment) concentrations of reactive nitrogen species (ASN) as a function of time. Overnight cultures were diluted 1/100 into 49 ml LB broth in a 250 ml flask and 1 ml of 5 mM ASN was added prior to incubation at 37°C with shaking at 200 rpm. Every hour 300 µl samples were removed and OD_{600} measured on a plate reader. Samples were diluted with LB broth if OD_{600} exceeded 0.7 and samples were taken every hour for 7 hrs.

2.5.4 Peroxynitrite killing assay

Peroxynitrite killing assay was performed with the same protocol as reactive oxygen species killing assay, with peroxynitrite (Merck) substituting for the presence of hydrogen peroxide or paraquat.

2.5.5 β-galactosidase assays

Overnight cultures were diluted 1/100 into fresh LB broth containing 100 µg ml^{-1} ampicillin prior to growth at 37°C, with shaking at 200 rpm, until an OD_{600} of 0.2-0.3 was reached. $CuSO_4$ or reactive nitrogen species were then added, to various concentrations, to the bacterial culture and incubation continued for 30 mins at room temperature and OD_{600} value was measured and recorded. β-galactosidase assays were performed essentially as previously described by Cavet *et al.* (2002). β-galactosidase activity was calculated as nmole *o*-nitrophenol (ONP) min^{-1} mg^{-1} protein, by measuring the absorbance of samples at OD_{414} and using the following equation:

$$\beta\text{-galactosidase activity} = 300 * \frac{(OD_{414(t)} - OD_{414\,(0)})}{13.68\ (T * V * OD_{595})}$$

Where T = reaction time (min), V = volume of culture used, 300 nmoles of ONP = 1 optical density unit at OD_{414}. $OD_{414(t)}$ = OD_{414} of the reaction terminated at time T, whereas $OD_{414(0)}$ = OD_{414} of the reaction terminated at time 0. 1/13.68 = OD_{595} of a S. Typhimurium culture that is equivalent to 1 mg ml^{-1} of protein.

2.6 *Salmonella* metal tolerance assays

2.6.1 Aerobic metal tolerance assays

Overnight cultures were grown in either fresh LB broth or minimal media and diluted 1/50 into fresh media, 2.475 ml of diluted culture was transferred to a 14 ml plastic tube (Falcon) and 25 µl of various concentrations of metal stocks, or H_2O, was added to each tube. Samples were incubated at 37°C with shaking at 200 rpm for 2-3 hrs to monitor growth in LB broth or 4-5 hrs to monitor growth in minimal media.

2.6.1.2 Anaerobic metal tolerance assays

A 1/50 dilution of an aerobic overnight culture was added to fresh LB broth or minimal media supplemented with 200 mM sodium fumarate and in which 0.4% (v/v) glucose was replaced with 0.4% (v/v) glycerol. Per sample, 49.95 ml of diluted overnight culture and 50 µl of a dissolved metal were gently mixed and uptaken within a 60 ml syringe (BD Plastipak), air bubbles were dispelled and the syringe sealed with a Luer-lock. LB broth cultures were grown for 3.5 hrs and minimal media cultures grown for 13 hrs statically at 37°C. Syringes

were shaken manually for 30 sec prior to a sample being squeezed out of the syringe into a 2 ml cuvettes and OD_{600} was measured in using a spectrophotometer.

2.6.1.3 Analyses of copper tolerance on solid media

Filter sterilised $CuSO_4$ was added to molten LB agar, to various concentrations, and mixed thoroughly prior to pouring into plates. Overnight cultures were serially diluted in PBS and 10 µl drops of the dilution series were then added to each LB agar plate supplemented with the different copper concentrations. Plates were incubated for 12-18 hrs at 37°C overnight. Anaerobic growth was performed by incubating plates within an anaerobic jar (Oxoid) with a sachet of AnaeroGen (Oxoid) for 48 hrs at 37°C. Plates were photographed using a CDC camera within a UV light transilluminator (UVITec UVIpro silver).

2.6.2 Determination of cellular metal quotas by ICP-MS analyses

2.6.2.1 S. Typhimurium cation content analyses

Overnight cultures were diluted 1/100 in 50 ml of minimal media within a 250 ml flask and cultures grown overnight at 37°C with shaking at 200 rpm for aerobic growth. For anaerobic growth, overnight bacterial cultures were diluted 1/100 in 100 ml minimal media supplemented with sodium fumarate and uptaken within two 60 ml syringes and grown overnight at 37°C. Following overnight incubation, 1 ml of culture was used to measure absorbance at OD_{600} and perform viable counts using Miles and Misra technique on LB agar plates. A further 1 ml of culture was used for protein quantification by Bradford assay (Section 2.5.4). The remaining 48 ml of culture was pelleted by centrifugation at 3,000 g for 20 min, the supernatant drained and the pellet re-suspended in 10 ml TE buffer (1% (v/v) 1M Tris pH 7.5, 0.2% (v/v) 0.5 M EDTA in mQH_2O) or 5 ml of TE buffer and the two samples combined for anaerobic cultures. Samples were re-pelleted by centrifugation at 3,000 g for 10 mins and the process was repeated three times with the final pellet being re-suspended in 10 ml mQH_2O. Pellets were dried at 80°C for 24 hrs followed by the addition of 0.5 ml 70% (v/v) suprapure nitric acid (Calbiochem) and incubated at 37°C with shaking at 200 rpm for 36 hrs. Samples were transferred to 1.5 ml microtubes and pelleted by centrifugation at 14,000 g for 5 min, 308 µl of supernatant was added to 9.69 ml mQH_2O. Samples were sent to Dr Paul Lythgoe (University of Manchester) who performed the ICP-MS analysis and returned the raw data.

2.6.2.2 Raw 264.7 macrophage cation content analyses

Macrophages were grown in a T162 cm^3 flask (Corning) until confluent (Section 2.7.1) and washed four times with 10 ml PBS prior to addition of 10 ml 0.9% (v/v) Triton-X and incubated at 37°C for 5 mins. Remaining adhered cells were re-suspended with a cell scraper (Greiner) and the flask contents transferred into a 15 ml tube and sent for ICP–MS analysis. A control flask was used to quantify viability of macrophages by combining with Trypan blue. Viable cells were counted using a haemocytometer chamber (Marienfield) and an Olympus CK40 light microscope (Trypan blue staining of live cells resulted in staining of the nucleus and dead cells stained entirely blue). The number of viable cells present within 16 μm^3 was determined in four separate repeats and the average number of viable cells ml^{-1} calculated.

2.6.3 Protein quantification assay

Protein concentrations were determined using the Bradford assay (Bradford 1976), 1 ml culture was pelleted by centrifugation at 14,000 g and re-suspended in TE buffer (as stated in section 2.6.2.1) for 10 mins then boiled for 10 mins. Samples were mixed at a 1:1 ratio with Coomassie brilliant blue. A protein standard curve was set up using bovine serum albumins (BSA) as instructed in the supplier's guidelines and absorbance measured at OD_{600} in a plate reader.

2.7 Culturing and infection of macrophages

2.7.1 Macrophage cell line growth and storage

The Raw 264.7 macrophage cell line (American type culture collection) was used with aliquots stored in liquid nitrogen. Cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS, Invitrogen) at 37°C, 5% CO_2 in a humidified incubator (Biohit galaxy) and all macrophage manipulations were performed within a class II safety cabinet unless stated otherwise. To resuscitate cells, a frozen aliquot was thawed on ice and transferred to a T25 cm^3 vented flask containing 5 ml DMEM and 10% (v/v) FBS. Macrophages were routinely passaged when 80-90% confluent into a new flask at a four-fold dilution and used for infection assays between passage 6 and 25. To passage cells were washed with 10 ml PBS, to remove metabolites and non-adhesive macrophages, before being re-suspended in 10 ml

DMEM with 10% (v/v) FBS with a cell scraper and transferred to a new T162 cm^3 flask containing 40 ml fresh DMEM and 10% (v/v) FBS. When > 10% of macrophages were non-adherent a fresh macrophage batch was thawed and used. Only early passage macrophages (< 4) were used to replenish frozen stocks. To replenish frozen stocks a confluent T162 cm^3 flask (approximately 2 x 10^7 macrophages) was re-suspended in 10 ml DMEM containing 10% (v/v) FBS, 10% (v/v) DMSO and 1 ml aliquots transferred into 1.5 ml cryogenic tubes. Samples were cooled -1°C per mins until -80°C within a Mr Frosty (Nalgene) containing iso-propanol. The following day the tubes were snap frozen in liquid nitrogen and stored in liquid nitrogen until required.

2.7.2 Bone marrow extraction of macrophage progenitor cells and differentiation

C57BL/6 mice legs were obtained from the in-house animal services (Biological Services Faculty). Skin and muscle tissue was removed from leg bones with forceps in sterile PBS. The upper and lower leg bones were cleaved at the knee joint and bone marrow was expelled by needle and syringe through administration of 2.5 ml DMEM per bone within the internal cavity, and collected in a 15 ml Falcon tube. Bone marrow progenitor cells were transferred to a T225 cm^3 and T75 cm^3 flask and 30 ml differentiation media was added prior to incubation. Differentiation media consists of a 70:30 ratio of fresh DMEM containing 10% (v/v) FBS and pre-used DMEM containing 10% (v/v) FBS from the fibroblast cell line L929 (ATCC); which releases macrophage colony stimulating factor during their growth. After three days, additional differentiation media was added equivalent to 20% of flask volume and cells were left for a further three days before progenitor cells differentiated into macrophages and were ready to be seeded for infection.

2.7.3 *Salmonella* intracellular survival assay

A confluent T162 cm^3 flask was passaged to remove DMEM, washed with PBS then re-suspended in DMEM containing 10% (v/v) FBS. Suspended macrophages were seeded at 1 x 10^5 macrophages per well (in 2 ml medium) in 6 well flat bottomed plates (Corning) and incubated for 18-24 hrs prior to bacterial challenge. Macrophage adherence and 90-95% confluency within wells was then confirmed using a light microscope (Olympus CK40 light microscope x60 magnification) immediately prior to infection. To determine viable cell numbers, macrophages were washed in PBS prior to resuspension in DMEM containing 10% (v/v) FBS and an aliquot diluted 1/10 in DMEM prior to mixing at a 1:1 ratio with Trypan

blue and viable macrophages counted as previously stated (section 2.6.2.2). For experiments using activated macrophages, 1000 U ml^{-1}mouse recombinant IFN-γ (Calbiochem) was added 12-16 hrs prior to infection and 6 hrs post seeding. For experiments under copper depleted conditions, DMEM containing 10% (v/v) FBS supplemented with 500 µM bathocuproine disulphonate (BCS) 12 hrs prior to use. iNOS activity was inhibited by the addition of 5 mM N^{g}-methyl L-arginine acetate (L-NMMA) 2 hrs post infection.

Competitive infection assays were performed. In brief, macrophages were washed with 0.5 ml PBS three times prior to the addition of 1 ml of PBS to each well. Bacteria were grown overnight in minimal media and OD_{600} determined and adjusted to within a 5% difference of culture with the lowest OD_{600} reading by addition of fresh media. For competitive infections, two bacterial were mixed together 1:1 and added to seeded macrophages to give an MOI of 10:1 (bacteria: macrophages) and bacteria brought into contact with macrophages by centrifugation at 300 g, 37°C for 5 min, to aid bacterial uptake (defined at time = 0h). Post centrifugation, plates were incubated for 1 hr at 37°C and then washed three times with 1 ml PBS and 2 ml of DMEM containing 100 µg ml^{-1} gentamycin was added to each well and incubation continued for 1 hr to kill any remaining extracellular bacteria. DMEM containing 100 µg ml^{-1} gentamycin was then replaced (time = 2 hours) with 2 ml fresh DMEM containing 20 µg ml^{-1} of gentamycin, to inhibit external bacterial replication, and plates incubated for the duration of infection. At specified time points DMEM medium was removed and cells washed four times with 1 ml of PBS and intracellular bacteria released by the addition of 0.5 ml 0.9% (v/v) Triton-X 100 and incubation for 5 mins at 37°C. Bacteria were re-suspended in the wells by pipetting and then serially diluted in PBS and plated using Miles and Misra technique onto both LB and LB chloramphenicol 10 µg ml^{-1} agar plates and grown at 37°C overnight. RNA extractions from infected cells were performed after washing macrophages four times with PBS then 0.5 ml of RNA protect (Qiagen) was added to each well followed by incubation for 5 mins at room temperature and infected macrophages were then re-suspended using a cell scrapper and 18 wells were combined together from which typical RNA extraction protocol was followed (section 2.2.11).

2.7.4 Measuring media nitrite levels

To measure nitrite release, and hence activation, from macrophages during infection assays, media from infection wells was transferred into a 1.5 ml microtube and pelleted by

centrifugation at 14,000 g for 1 min. Nitrite was quantified as previously stated (section 2.5.3) with Griess reagent.

2.7.5 IL-1β extraction and detection

Infections were performed as previously stated with SL1344 in macrophages pre-treated with and without IFN-γ. At determined time points macrophages were lysed with 0.5 ml 0.9% (v/v) Triton-X 100. Lysate was pelleted by centrifugation at 14,000 g for 10 mins and supernatant was transferred into a microtube and stored at -20°C until required. ELISA was performed using a mouse IL-1β ELISA kit (RnD) following supplier's instructions. IL-1β levels were quantified using a standard curve of known IL-1β levels.

2.7.6 Immunofluorescent staining and imaging

Coverslips were sterilised by dipping in 100% (v/v) ethanol and flame dried, then placed within a well of a 6 well plate. Macrophages were passaged normally and seeded into wells containing coverslips. Infection protocol was performed as above and at various time points post infection, macrophages were washed four times with 1 ml PBS and fixed to coverslips by the addition of 1 ml 3% (v/v) paraformaldehyde, incubated at room temperature for 20 mins and then washed with 1 ml PBS three times, with the second PBS wash containing 20 mM glycine pH 8.5. Macrophages were permeabilized with 1 ml 0.1% (v/v) Triton X-100 in TBS (50 mM Tris, 150 mM NaCl pH 7.4) for 10 mins at room temperature and washed three times with 1 ml PBS then stored in PBS at 4°C until all time points were finished. Coverslips were blot dried with tissue paper and incubated on top of a 20 µl drop of Image-iT FX signal enhancer (Molecular Probes) on a piece of parafilm in a humid environment at room temperature for 30 mins. Permeabilised macrophages were then washed with TBS three times and then blocked with permeabilization blocking buffer for 30 mins at room temperature. Blocking buffer contained: 1% (v/v) fish gelatine, 0.1% (v/v) Triton X-100, 10% (v/v) normal goat serum, 1X TBS in mQH_2O. To stain bacteria, rabbit anti-O4 serum stain (Merck) was diluted 1/500 in blocking buffer and 50 µl was placed onto a piece of parafilm and the coverslip was placed face down onto the drop and incubated in darkness for 1 hr at room temperature. Macrophages were then washed three times with TBS and then stained with 50 µl of secondary antibody (AlexaFluor 555 goat anti-rabbit, Invitrogen) diluted 1/2000 in blocking buffer), as described for the primary antibody stain. Subsequently, coverslips were incubated in TBS containing 1x DAPI for 10 mins at room temperature on a shaking

platform and washed three times in TBS then three times in mQH_2O. A 10 µl drop of mounting media was added to a slide and the coverslip with stained fixed cells placed face down on the mounting media and left to set at room temperature for 2 hrs in darkness, and then stored at 4°C. Staining was visualised using a fluorescent microscope with the confocal setting (Nicon C1 inverted with TIRF), DAPI was excited at 360-370 nm and emissions measured at 420-460 nm, Texas red (secondary antibody) was excited at 560-550 nm and emission measured at 645-750 nm.

Chapter 3

To investigate the role of the S. Typhimurium copper homeostatic systems in providing protection against reactive oxygen and nitrogen species in the presence and absence of copper

3.1 Introduction

S. Typhimurium is an intracellular pathogen residing within macrophages or macrophage like cells during systemic infection. Intracellular S. Typhimurium are exposed to a number of antimicrobial agents including reactive oxygen and nitrogen species, copper and the endocytic pathway. Several intracellular pathogens such as S. Typhimurium and *M. tuberculosis* are able to avoid degradation through the endocytic pathway by inhibiting the fusion of lysosomes to the phagosome (Buchmeier and Heffron 1991). Nonetheless, pathogens are still exposed to antimicrobial mechanisms in the phagosomal compartment.

Reactive oxygen species are primarily utilised to kill pathogens by protein, lipid and DNA damage. Reactive nitrogen species also target protein, lipids and DNA, but inhibit the growth of intracellular pathogens rather than destroying the pathogen. Reactive oxygen species are produced within one hour of phagocytosis of a pathogen whereas reactive nitrogen species are produced approximately 8 hours after phagocytosis (Eriksson *et al.* 2003). Peroxynitrite is formed from the combination of superoxide and nitric oxide, although its classification as a separate stress to reactive oxygen species is debated.

Recent publications have identified copper as an antimicrobial agent within macrophage phagosomes (White *et al.* 2009, Achard *et al.* 2012, Osman *et al.* 2010). If unregulated copper can induce damage indirectly by catalysing Fenton chemistry to release hydroxyl radicals, this contributing to reactive oxygen mediated killing, or directly disrupt metalloproteins such as iron sulphur clusters (Macomber and Imlay 2009). Reactive nitrogen species have also been identified to liberate bound copper from the metallothionine MymT within *M. tuberculosis* highlighting the potential that reactive nitrogen species could increase copper levels inside an intracellular pathogen. Thus, the combined actions of copper and reactive nitrogen species may contribute to pathogen killing. The presence of copper within the *Salmonella* containing vacuole is detected 12 hours post infection, consequently at a similar timing to the production of reactive nitrogen species (Osman *et al.* 2010, Eriksson *et al.* 2003).

3.2 Reactive oxygen species

3.2.1 Copper increases the toxicity of hydrogen peroxide

Hydrogen peroxide can form hydroxyl radicals in the presence of a redox agent. Iron is typically given as the example but copper is also capable of redox actions to potentiate a greater conversion of hydrogen peroxide into hydroxyl radicals as shown below (Fenton 1894).

$Cu^{+} + O_2 \rightarrow Cu^{2+} + O_2^{-}$

$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + OH^{-} + OH^{\cdot}$

Without the presence of a redox agent hydrogen peroxide can be converted into oxygen and water as shown below by catalase, alkyl hydroperoxide reductase or glutathione peroxidase activity.

$2H_2O_2 \rightarrow 2H_2O + O_2$

Hydrogen peroxide can readily diffuse into S. Typhimurium due to its uncharged status, consequently when S. Typhimurium is present in a hydrogen peroxide rich environment it is highly susceptible to reactive oxygen species mediated damage. The presence of hydrogen peroxide and copper together is proposed to be particularly toxic due to Fenton chemistry. To investigate whether or not copper increases hydrogen peroxide toxicity to S. Typhimurium endpoint hydrogen peroxide and copper survival assays were performed with SL1344. An overnight culture was diluted 1/100 into fresh LB and incubated in the presence of hydrogen peroxide with and without supplementation of $CuSO_4$ (figure 3.1). Copper and hydrogen peroxide alone were capable of killing S. Typhimurium but when the two were combined significantly greater killing occurred ($p < 0.05$). S. Typhimurium incubated with copper had a viable count of $1x10^7$, with hydrogen peroxide $5.5x10^6$ and the combination of copper and hydrogen peroxide $1.1x10^6$. This is consistent with previous work that suggests copper increases the toxicity of hydrogen peroxide toward bacteria (Fenton 1894 and White *et al.* 2009).

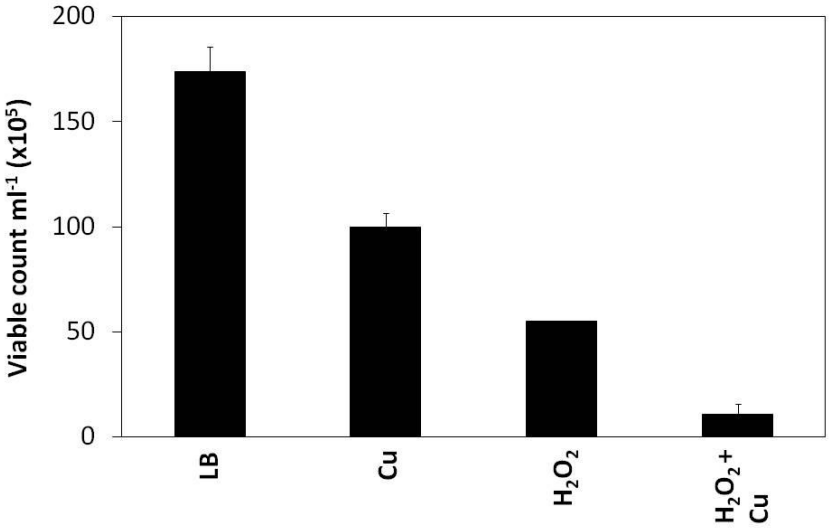


Figure 3.1 Copper increases toxicity of hydrogen peroxide towards S. Typhimurium

An overnight culture of SL1344 (black) was diluted 1/100 into fresh LB and supplemented with 1 mM $CuSO_4$ and/or 50 µM H_2O_2 where stated and incubated at 37°C for 2 hours. Bacteria were then serially diluted in PBS and quantified by plating onto LB agar by the method of Miles and Misra and grown at 37°C statically overnight. Data points represent the mean of three independent repeats performed in triplicate, error bars represent standard error.

3.2.2 Copper homeostasis mutants have similar hydrogen peroxide tolerance to wildtype S. Typhimurium

Having confirmed that copper increases the toxicity of hydrogen peroxide toward S. Typhimurium, the tolerance of copper homeostasis mutants to hydrogen peroxide, in the presence and absence of sub-lethal concentrations of copper was examined. It has previously been identified that S. Typhimurium lacking the two copper exporting P_{1B}-type ATPases CopA and GolT(*ΔcopA/ΔgolT*) accumulate 25 fold greater copper levels than SL1344 when grown in sub-lethal concentrations of copper (Osman *et al.* 2010). Hence, may be more sensitive to hydrogen peroxide than wildtype cells due to the increased potential for Fenton chemistry.

Endpoint hydrogen peroxide and/or copper survival assays were performed with SL1344, *ΔcopA/ΔgolT*, *ΔgolB* and *ΔcueO/ΔcueP* (figure 3.2). No significant difference was seen between copper homeostasis mutants when exposed to hydrogen peroxide in the presence and absence of copper. As previously (figure 3.1), copper increased the toxicity of hydrogen peroxide to S. Typhimurium and a similar reduction was observed for the copper homeostasis mutants. Increased killing from the addition of copper was seen at 50 μM and 100 μM hydrogen peroxide with the largest difference at 100 μM. When incubated with 100 μM hydrogen peroxide SL1344 had a viable count of $5.78x10^4$ and with the addition of 0.25 mM $CuSO_4$ a viable count of $5.54x10^3$, a difference of more than one log.

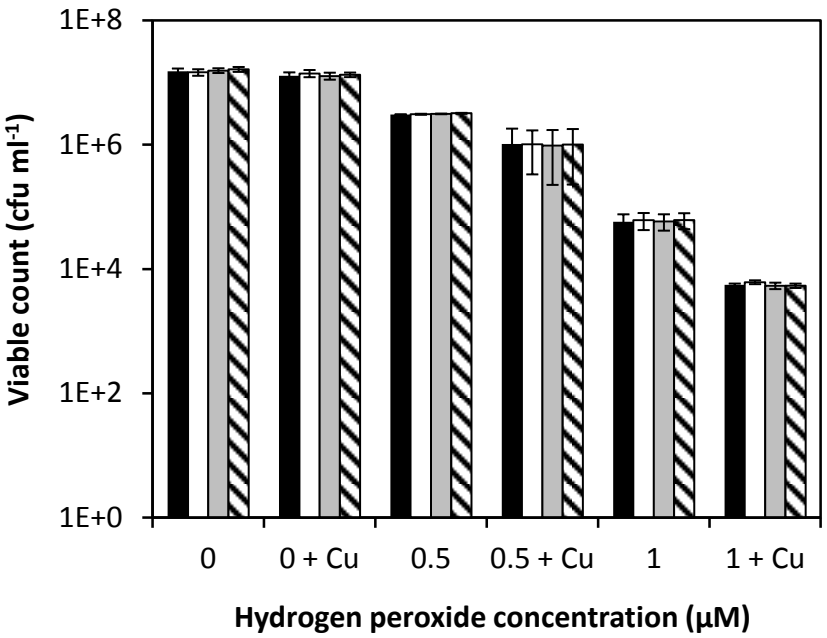


Figure 3.2 Copper homeostasis mutants of S. Typhimurium have similar tolerance to hydrogen peroxide as wildtype in the presence and absence of copper

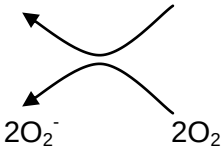
Overnight cultures of SL1344 (black), *ΔcopA/ΔgolT* (white), *ΔgolB* (grey) and *ΔcueO/ΔcueP* (diagonal lines) were diluted 1/100 into fresh LB media in the presence of 0 µM, 50 µM and 100 µM H_2O_2 in the presence and absence of 0.25 mM $CuSO_4$ and incubated at 37°C for 2 hours. Bacteria were serially diluted in PBS and quantified by plating onto LB agar by the method of Miles and Misra and grown at 37°C statically overnight. The following day colonies were counted and converted to cfu ml^{-1}. Data points represent the mean of three independent repeats performed in triplicate, error bars represent standard error.

The lack of a tolerance difference between the survival of copper homeostasis mutants and SL1344 to hydrogen peroxide in the presence and absence of sub-lethal copper indicates that hydrogen peroxide killing is not influenced by bacterial cellular copper contents or ability to detoxify copper. Indeed, despite the increased levels of copper within *ΔcopA/ΔgolT* no reduction on hydrogen peroxide tolerance was detected. S. Typhimurium is able to complex large quantities of copper in a form that is unable to catalyse Fenton chemistry. As the presence of copper gave increased killing than incubation with hydrogen peroxide alone, copper is most likely catalysing Fenton chemistry outside of S. Typhimurium, as such hydroxyl radicals may then either directly attack the outer membrane of S. Typhimurium or diffuse through porins and attack periplasmic molecules. The creation of hydroxyl radicals outside of S. Typhimurium would explain the killing seen across all strains, regardless of copper homeostatic capabilities, in the presence of copper and hydrogen peroxide.

3.2.3 S. Typhimurium copper homeostasis mutants have similar paraquat tolerance

An alternate reactive oxygen species generator was investigated, paraquat, that generates superoxide within a host cell. Paraquat receives electrons from NADPH forming a paraquat radical that reduces oxygen into superoxide returning paraquat to its prior oxidation state, as shown below.

$C_{12}H_{14}Cl_2N_2^{2+}$ (paraquat) + $2e^-$ → $C_{12}H_{14}Cl_2N_2$ (paraquat free radical)



The tolerance of wildtype S. Typhimurium and various copper homeostatic mutants to paraquat was examined in the presence and absence of copper. SL1344 was incubated in the presence and absence of paraquat and copper (figure 3.3). Incubation of SL1344 in the presence of 50 mM paraquat had a viable count of 1.35 $x10^7$, with 1 mM $CuSO_4$ a viable count of 2.72 $x10^7$ and the combination of paraquat and $CuSO_4$ gave a viable count of 1.30 $x10^7$. No difference was seen between the viable counts for S. Typhimurium exposed to paraquat, copper or paraquat and copper. Hence, copper does not increase the potency of paraquat toward S. Typhimurium tolerance. The tolerance of copper homeostasis mutants to paraquat, in the presence and absence of sub-lethal concentrations of copper was examined (figure 3.4). No significant difference in tolerance to superoxide was seen between copper homeostasis mutants and SL1344.

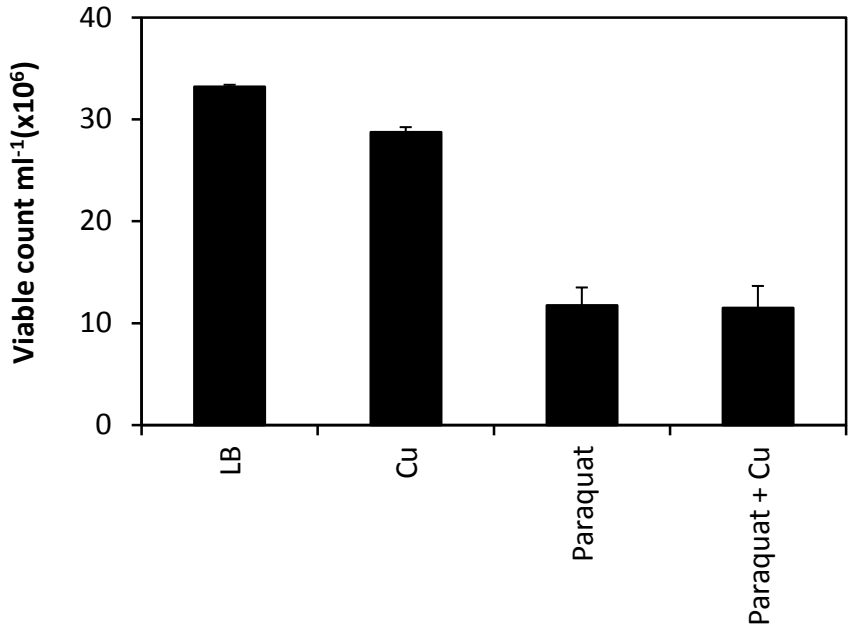


Figure 3.3 Copper does not alter S. Typhimurium tolerance to paraquat

An overnight culture of SL1344 (black) was diluted 1/100 into fresh LB and supplemented with 1 mM $CuSO_4$ and 50 mM paraquat where stated and incubated at 37°C for 2 hours. Bacteria were serially diluted in PBS and quantified by plating onto LB agar by the method of Miles and Misra and grown at 37°C statically overnight. Data points represent the mean of three independent repeats performed in triplicate, error bars represent standard error.

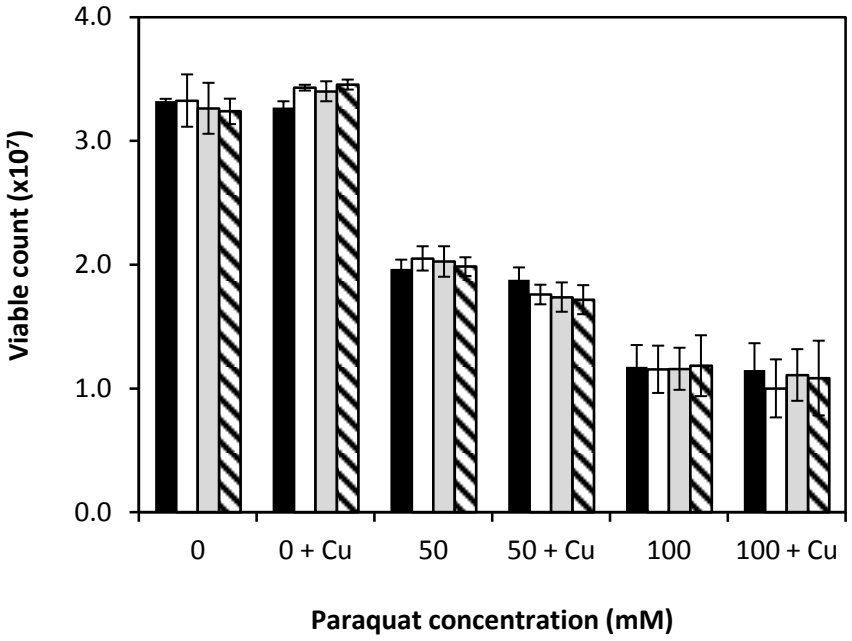


Figure 3.4 Copper homeostasis mutants of S. Typhimurium have similar tolerance to paraquat as SL1344 in the presence and absence of copper

Overnight cultures of SL1344 (black), *ΔcopA/ΔgolT* (white), *ΔgolB* (grey) and *ΔcueO/ΔcueP* (diagonal lines) were diluted 1/100 into fresh LB media in the presence of 0, 50 mM and 100 mM paraquat with and without 0.25 mM $CuSO_4$ and incubated at 37°C for 2 hours. Bacteria were serially diluted in PBS and quantified by plating onto LB agar by the method of Miles and Misra and grown at 37°C statically overnight. The following day colonies were counted and converted to cfu ml^{-1}. Data points represent the mean of three independent repeats performed in triplicate, error bars represent standard error.

3.2.4 S. Typhimurium copper homeostasis mutants do not have any difference in tolerance to reactive oxygen species generated by xanthine oxidase

Xanthine oxidase (XOD) catalyses the formation of hydrogen peroxide and superoxide by the oxidation of xanthine as shown below (Kelley *et al.* 2010). The production of either hydrogen peroxide or superoxide is dependent upon O_2 concentration within the surrounding environment. Superoxide production is proportional to oxygen concentration available; at atmospheric O_2 concentration (21%), approximately four molecules of hydrogen peroxide are produced per atom of superoxide (Kelley *et al.* 2010). The addition of hydrogen peroxide to a bacterial culture supplies a high initial concentration of reactive oxygen species decreasing with time as Fenton chemistry is catalysed. The use of xanthine oxidase maintains a consistent concentration of hydrogen peroxide and superoxide throughout the experiment.

$C_5H_4N_4O$ (Hypoxanthine) + $2H_2O$ + O_2 →$C_5H_4N_4O_2$ (Xanthine) + H_2O_2 + $2H^+$ + O_2^-

$C_5H_4N_4O_2$ (Xanthine) + $3H_2O$ + $2O_2$ → $C_5H_4N_4O_3$ (Uric acid) + $2H_2O_2$ + $2H^+$ + O_2^-

Xanthine oxidase, unlike paraquat, generates reactive oxygen species outside of S. Typhimurium. To confirm xanthine oxidase production of superoxide xanthine oxidase and hypoxanthine were mixed together and superoxide production quantified by addition to nitroblue tetrazolium (figure 3.5A). Upon confirming xanthine oxidase production of reactive oxygen species, the tolerance of wildtype S. Typhimurium to xanthine oxidase generated reactive oxygen species in the presence and absence of copper was examined (figure 3.5B). The presence of copper did not influence S. Typhimurium tolerance to xanthine oxidase generated reactive oxygen species. The tolerance of copper homeostasis mutants to xanthine oxidase generated reactive oxygen species was examined (figure 3.6). No significant difference was seen between copper homeostasis mutants.

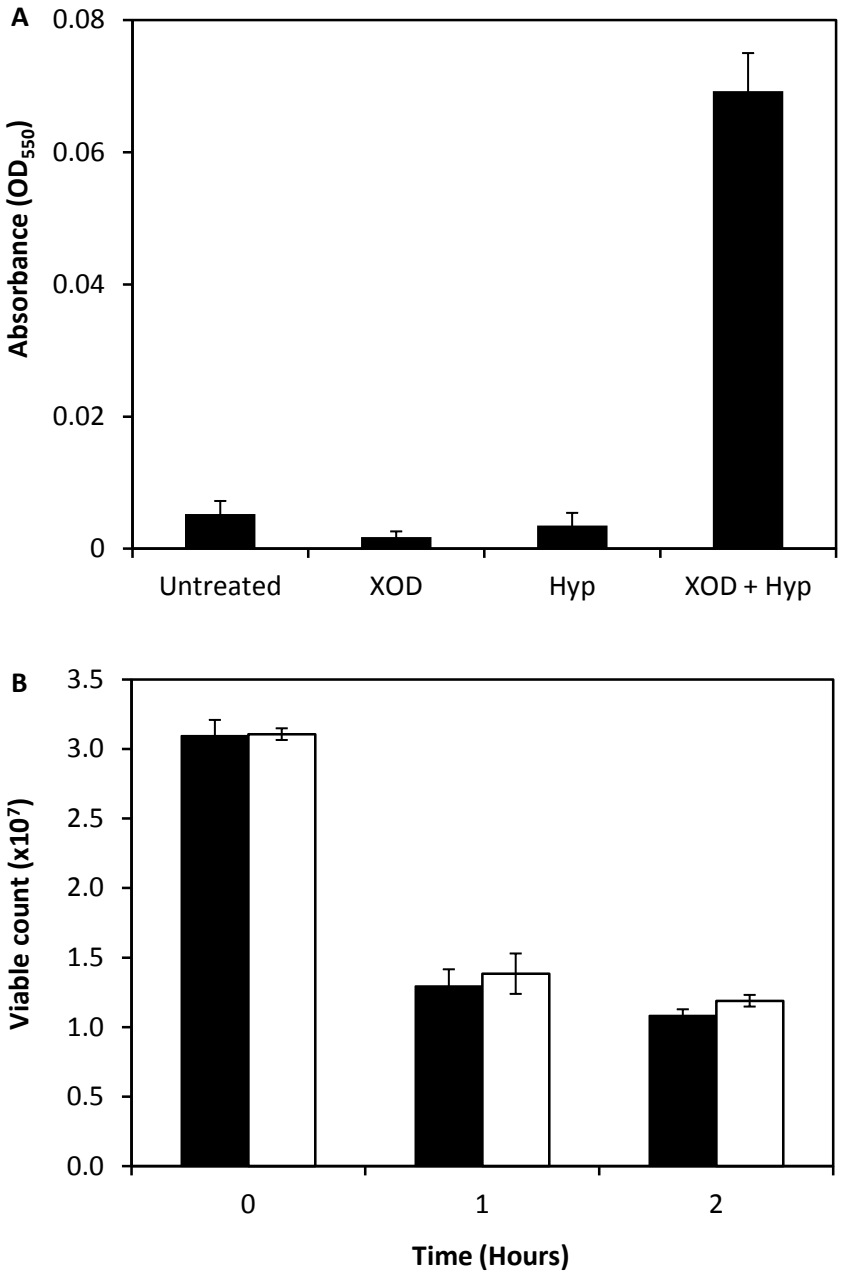


Figure 3.5 Copper does not enhance killing of S. Typhimurium by xanthine oxidase

(A) Hydrogen peroxide production was quantified by the addition of XOD and hypoxanthine as indicated to PBS and then mixed 1:1 with 10 mM nitroblue tetrazolium and incubated at room temperature for 10 mins. Absorbance at OD_{550} was measured on a plate reader. (B) An overnight culture of SL1344 was diluted 1/100 into fresh LB with 5 units xanthine oxidase and 25 μM hypoxanthine and supplemented with (black) and without (white) 1 mM $CuSO_4$ and incubated at 37°C 200 rpm for 2 hours. Bacteria were serially diluted in PBS and quantified by plating onto LB agar by the method of Miles and Misra and grown at 37°C statically overnight. Data points represent the mean of three independent repeats performed in triplicate, error bars represent standard error.

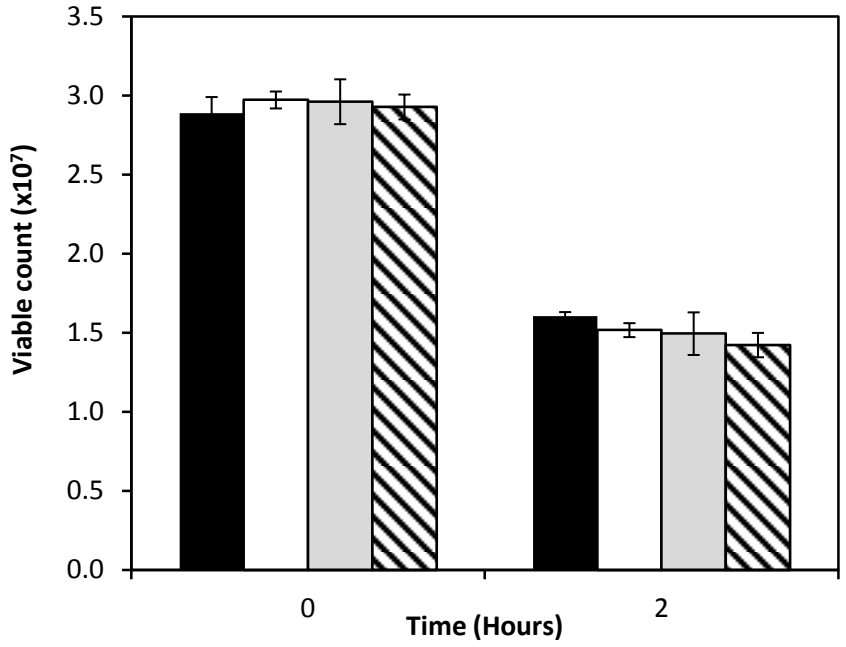


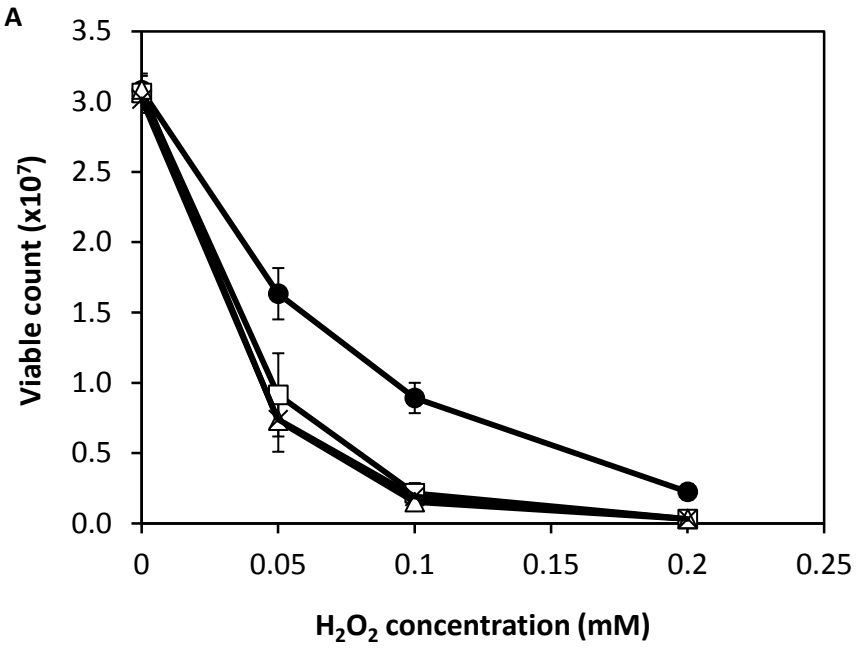
Figure 3.6 Copper homeostasis mutants of S. Typhimurium have similar tolerance to xanthine oxidase generated reactive oxygen species as SL1344

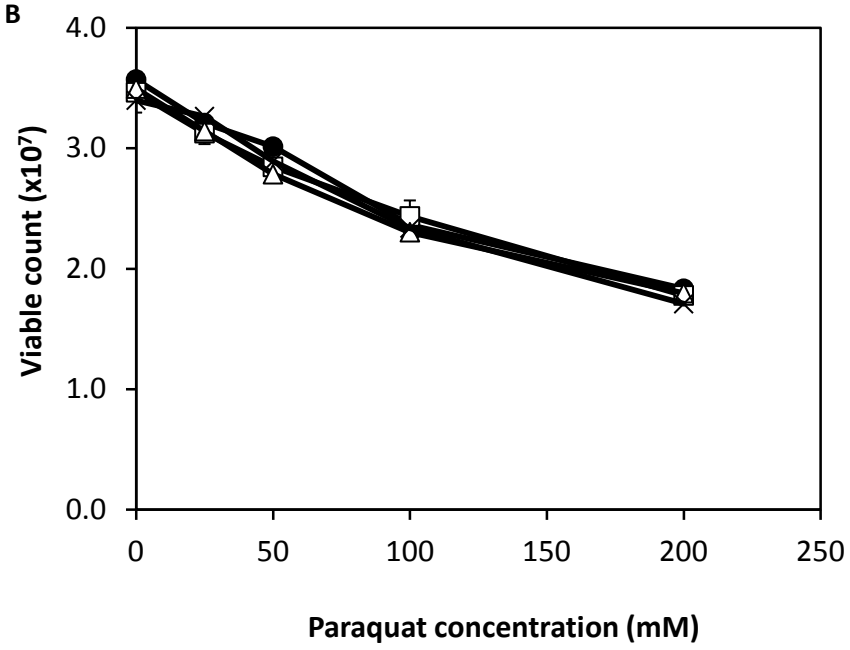
Overnight cultures of SL1344 (black), *ΔcopA/ΔgolT* (white), *ΔgolB* (grey) and *ΔcueO/ΔcueP* (diagonal lines) were diluted 1/100 into fresh LB and supplemented with 5 units xanthine oxidase and 25 μM hypoxanthine where stated and incubated at 37°C for 2 hours. Bacteria were serially diluted in PBS and quantified by plating onto LB agar by the method of Miles and Misra and grown at 37°C statically overnight. Data points represent the mean of three independent repeats performed in triplicate, error bars represent standard error.

It has been suggested (A. McEwan, University of Queensland, personal communication) that a subtle phenotype for sensitivity to reactive oxygen species could be masked by the action of catalase. Catalase converts hydrogen peroxide into water and oxygen thus reducing hydrogen peroxide toxicity and potentially preventing Fenton chemistry. Catalase has been identified to have an extremely high working efficiency during *in vitro* assays showing no saturation even when exposed to substantially high hydrogen peroxide levels (Lledias *et al.* 1998). A catalase mutant (*katG*) of S. Typhimurium was made by insertional mutagenesis as previously described in Datsenko and Wanner (2000) to test whether or not copper homeostasis mutants show increased sensitivity to reactive oxygen species when KatG activity is absent.

3.2.5 An S. Typhimurium *katG* mutant has decreased tolerance to hydrogen peroxide than SL1344 but no difference in tolerance to paraquat and xanthine oxidase generated reactive oxygen species

After creating an S. Typhimurium *katG* mutant the *katG* mutation was moved by P22 phage transduction into a *copA/golT* double mutant and a $sodC_I/sodC_{II}$ double mutant. Reduced catalase activity was confirmed by another member of the laboratory group (K. Muddiman, private communication). A $\Delta sodC_I/\Delta sodC_{II}/\Delta katG$ strain was investigated due to having reduced reactive oxygen species tolerance to both hydrogen peroxide (*ΔkatG*) and superoxide ($\Delta sodC_I/\Delta sodC_{II}$). To identify if catalase makes a significant contribution to detoxification of oxidative stress, the tolerance of the *katG* mutant strains toward hydrogen peroxide, paraquat and xanthine oxidase was tested (figure 3.7). A *katG* mutant had increased sensitivity to hydrogen peroxide but the addition of *copA/golT* or $sodC_I/sodC_{II}$ mutations did not further reduce hydrogen peroxide tolerance of S. Typhimurium. No difference was seen between SL1344 and mutant strains for paraquat and xanthine oxidase generated reactive oxygen species tolerance.


A
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Viable count (x10⁷)
0
0.05
0.1
0.15
0.2
0.25
H2O2 concentration (mM)


B
4.0
3.0
2.0
1.0
0.0
Viable count (x10⁷)
0
50
100
150
200
250
Paraquat concentration (mM)

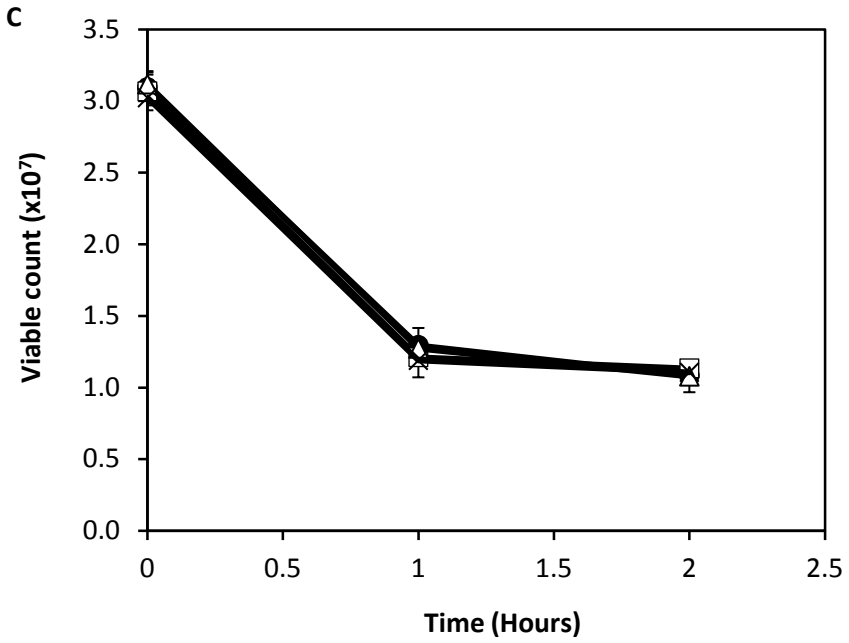


Figure 3.7 KatG provides S. Typhimurium with protection against hydrogen peroxide but not paraquat or xanthine oxidase generated reactive oxygen species

Overnight cultures of SL1344 (●), *ΔkatG* (□), *ΔcopA/ΔgolT/ΔkatG* (Δ) and *ΔsodC$_I$/ΔsodC$_{II}$/ΔkatG* (x) were diluted 1/100 into fresh LB and supplemented with (A) hydrogen peroxide, (B) paraquat and incubated at 37°C for 2 hours, or (C) 5 units xanthine and 25 μM hypoxanthine and incubated at 37°C at 200 rpm for 2 hours. Bacteria were serially diluted in PBS and quantified by plating onto LB agar by the method of Miles and Misra and grown at 37°C statically overnight. Data points represent the mean of three independent repeats performed in triplicate, error bars represent standard error.

A *katG* mutant showed reduce tolerance to hydrogen peroxide but not paraquat or xanthine oxidase, thus the reduced tolerance of S. Typhimurium is consistent with the loss of catalase activity and increased hydrogen peroxide potency. Catalase functions by detoxifying hydrogen peroxide into water and oxygen. Xanthine oxidase produces hydrogen peroxide and superoxide throughout the two hour incubation. Xanthine oxidase produces regular but low concentrations of approximately 1.6 µM hydrogen peroxide and 0.4 µM superoxide per two minutes (McCord and Fridovich 1969). Consequently for the duration of the experiment approximately 96 µM of hydrogen peroxide and 24 µM superoxide were produced. For the experiment using hydrogen peroxide, the lowest concentration used was 50 µM and thus was added entirely at the start of the experiment, this was too great a concentration for the remaining catalase enzymes to detoxify within S. Typhimurium. However, the alternate catalases, KatN and KatE, appear capable of detoxifying the lower concentration of hydrogen peroxide produced by xanthine oxidase throughout the 2 hour experiment duration. No difference in tolerance was seen between SL1344, *ΔkatG*, *ΔcopA/ΔgolT/ΔkatG* and *ΔsodC$_I$/ΔsodC$_{II}$/ΔkatG* for paraquat and xanthine oxidase generated reactive oxygen species. This is consistent with previous data, confirming that *ΔcopA/ΔgolT* does not have any difference in tolerance to reactive oxygen species than SL1344 and that KatG catalase activity is not preventing detection of a potential phenotype. The lack of increased killing of *ΔsodC$_I$/ΔsodC$_{II}$/ΔkatG* indicates that superoxide produced by paraquat or xanthine oxidase was detoxified by cytoplasmic superoxide dismutase enzymes (SodA and SodB). Due to the absence of SodC$_I$ and SodC$_{II}$ greater concentrations of superoxide would be expected to reach the cytosol and consequently expression of both *sodA* and *sodB* would increase.

Deletion of copper homeostasis genes did not increase susceptibility to reactive oxygen species killing. Despite disruptions of the copper homeostatic mechanisms in S. Typhimurium, increased copper accumulation in *ΔcopA/ΔgolT*, copper does not increase the potency of reactive oxygen species in the cells. Internalised copper is unable to function as a redox agent and catalyse the production of hydroxyl radicals, indicating that copper is retained in a complexed, inaccessible form in S. Typhimurium. Although hydrogen peroxide toxicity is increased by the presence of copper this was not the case for superoxide generators paraquat and xanthine oxidase. Analysis of a *katG* mutation strains identified increased sensitivity to hydrogen peroxide but not paraquat or xanthine oxidase, consistent with some loss of catalase activity and retention of superoxide tolerance. The lack of change in reactive oxygen species tolerance by *ΔcopA/ΔgolT* in the presence or absence of sub-lethal copper indicates that the reduced survival seen in macrophage intracellular survival assays does not relate to an increase in reactive oxygen species toxicity.

3.3 Reactive nitrogen species

Reactive nitrogen species prevent replication of internalised pathogens enabling the immune response to become augmented and recruit further lymphocytes to the infection site. Reactive nitrogen species refer to a larger range of molecules than reactive oxygen species. The ability of different reactive nitrogen species to modify proteins, lipids and nucleic acid varies upon the specific reactive nitrogen species. Previously it has been identified that reactive nitrogen species are capable of liberating bound metals from metalloproteins. This was identified for the copper binding metallothionine MymT within *M. tuberculosis* (Gold *et al.* 2008). Reactive nitrogen species were investigated to identify if they could increase copper toxicity toward S. Typhimurium copper homeostatic mutants. If reactive nitrogen species are capable of liberating bound copper within S. Typhimurium this would be anticipated to have several detrimental effects for the bacterium.

3.2.1 Copper does not increase the potency of reactive nitrogen species toward S. Typhimurium

Due to the diverse nature of reactive nitrogen species three reactive nitrogen species generators were used: ASN, GSNO and NOC5/7, each developing reactive nitrogen species through a different mechanism. ASN consists of sodium nitrite buffered at an acidic pH that breaks down into a range of reactive species as shown below:

$NO_2^- + H^+ \leftrightarrow HNO_2$

$2HNO_2 \leftrightarrow N_2O_3 + H_2O$

$N_2O_3 \leftrightarrow NO^{\cdot} + NO_2$

ASN gives a mixture of reactive nitrogen species and was the least clean reactive nitrogen species generator utilised. GSNO and NOC5/7 are specialised compounds that differ in delivery of reactive nitrogen species stress. GSNO is a nitrosylating agent that does not generate nitric oxide but nitrosylates another ligand transferring an $NO^{\cdot}$ group. NOC5/7 compounds generate nitric oxide directly at specific half life times, NOC-5 has a long half life time of 93 mins whereas NOC-7 has a short half life time of 10 mins. The reactions of GSNO and NOC5/7 are summated below.

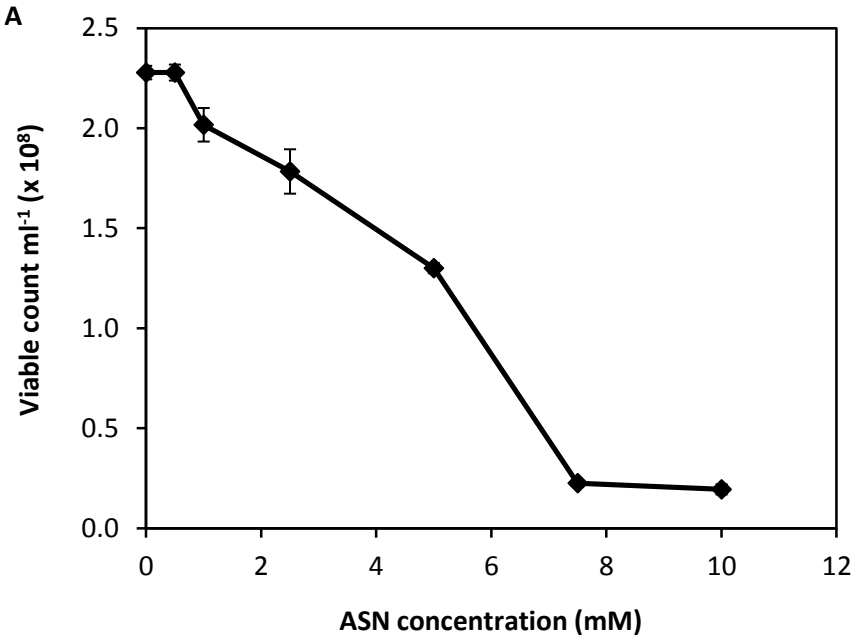
RS (thiol ligand) + $C_{10}H_{16}N_4O_7S$ (GSNO) → RSNO (nitrosylated ligand) + GSH (glutathione)

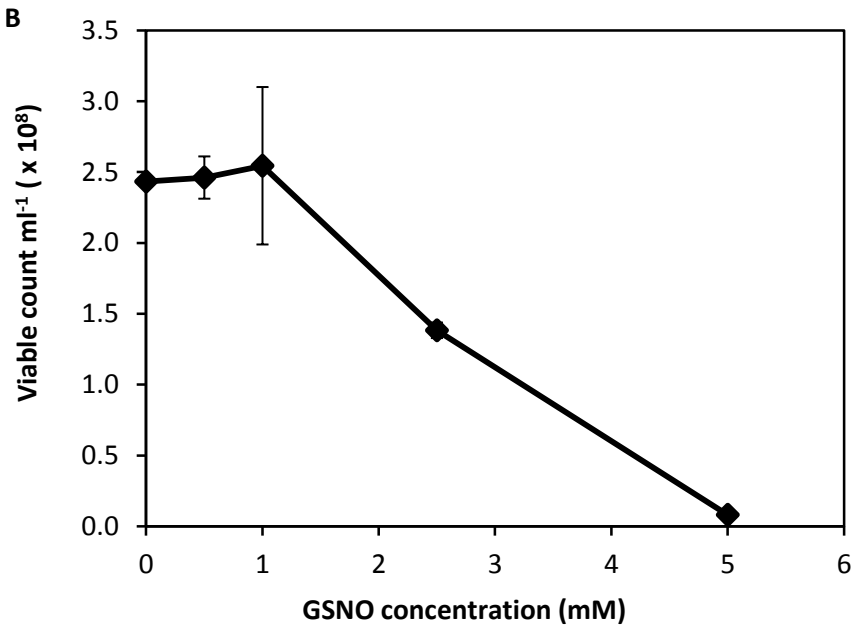
$C_6H_{16}N_4O_2$ (NOC-5) → $C_6H_{16}N_3O$ + $NO^{\cdot}$

$C_5H_{14}N_4O_2$ (NOC-7) → $C_5H_{14}N_3O$ + $NO^{\cdot}$

To determine the effective concentrations to limit growth of S. Typhimurium, titrations were initially performed with SL1344 in the presence of ASN, GSNO and NOC-5 and NOC-7 (figure 3.8).

Exposure of S. Typhimurium to increasing concentrations of reactive nitrogen species generators identified levels that inhibited growth of SL1344. For further experiments a reactive nitrogen species generator concentration of; 5 mM ASN, 5 mM GSNO and 2 mM NOC5/7 5/7 were utilised as gave a decrease in SL1344 growth. To analyse if copper can increase toxicity of reactive nitrogen species to S. Typhimurium, a copper titration was performed with the chosen inhibitory concentrations of reactive nitrogen species (figure 3.9). No difference in growth was seen between S. Typhimurium viable counts with and without the presence of reactive nitrogen species generators at increasing copper concentrations. Hence copper does not appear to increase the potency of reactive nitrogen species towards S. Typhimurium.


A
Viable count ml-1 (x 10^8)
2.5
2.0
1.5
1.0
0.5
0.0
0
2
4
6
8
10
12
ASN concentration (mM)


B
Viable count ml-1 (x 10^8)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
1
2
3
4
5
6
GSNO concentration (mM)

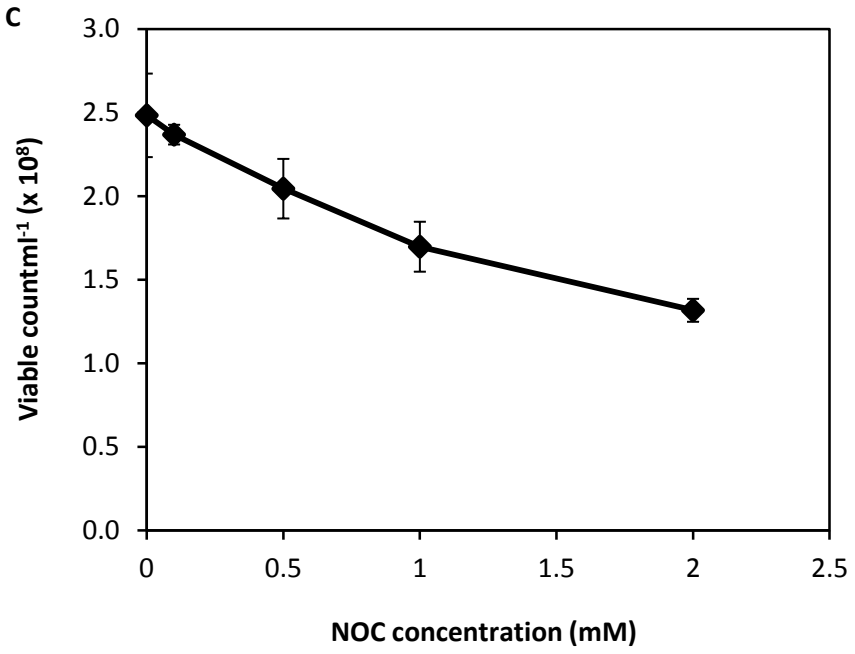
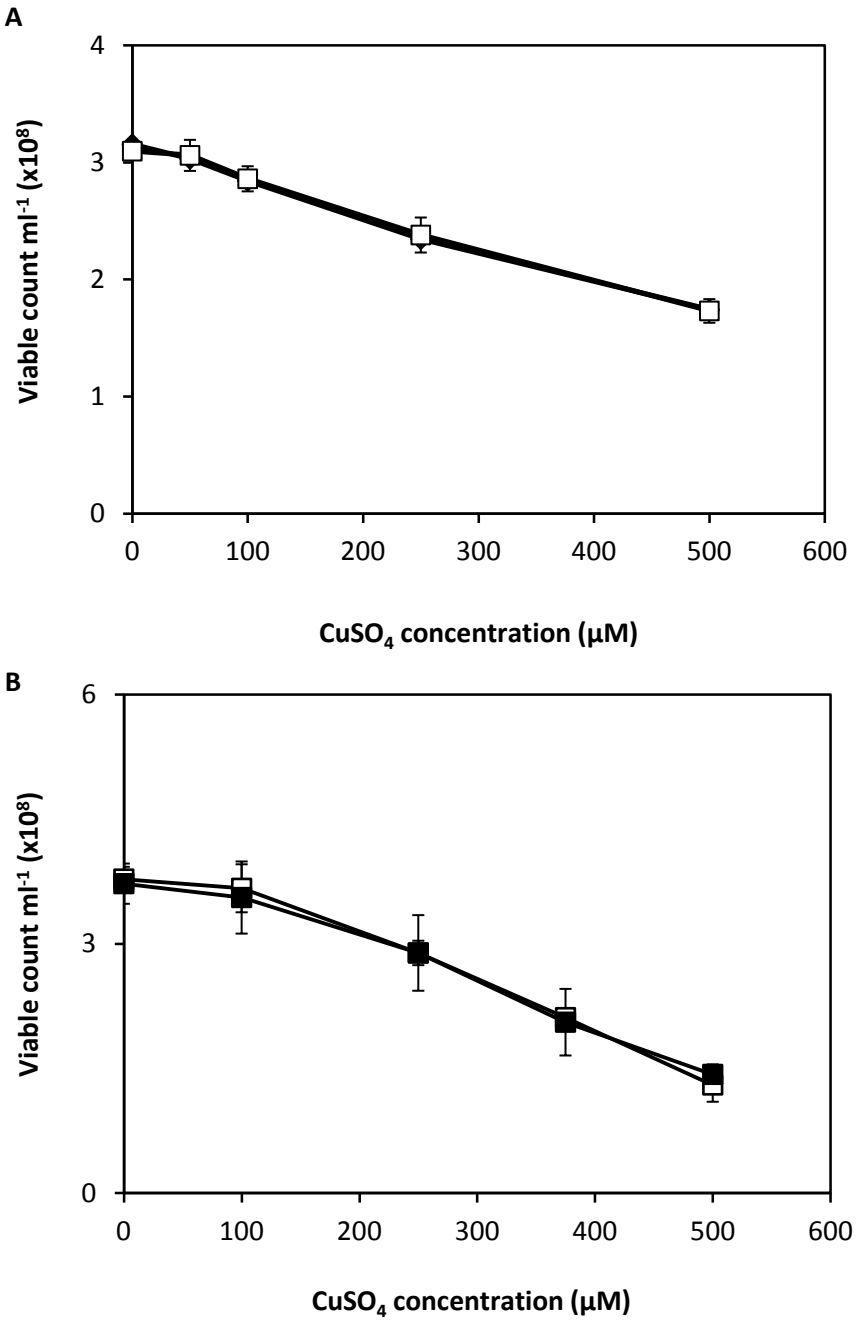


Figure 3.8 Growth of SL1344 in the presence of increasing concentrations of ASN, GSNO and NOC5/7 5/7

An overnight culture of SL1344 was diluted 1/1000 into fresh LB media in the presence and absence of (A) ASN, (B) GSNO and (C) NOC5/7 5/7 and incubated at 37°C for 7 hours. Bacteria were serially diluted in PBS and quantified by plating onto LB agar by the method of Miles and Misra and grown at 37°C statically overnight. The following day colonies were counted and converted to cfu ml^{-1}. Data points represent the mean of one repeat performed in triplicate, error bars represent standard error.


A
Viable count ml-1 (x108)
4
3
2
1
0
0
100
200
300
400
500
600
CuSO4 concentration (µM)
B
Viable count ml-1 (x108)
6
3
0
0
100
200
300
400
500
600
CuSO4 concentration (µM)

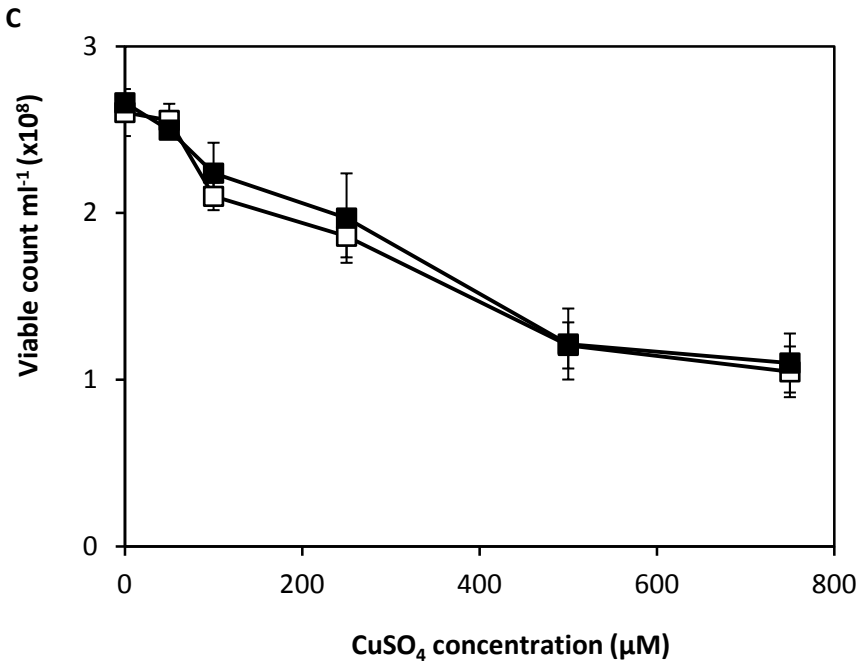


Figure 3.9 Copper does not increase the potency of ASN, GSNO or NOC5/7 toward S. Typhimurium

An overnight culture of SL1344 was diluted 1/1000 into fresh LB media in the presence of increasing concentrations of $CuSO_4$, with (□) and without (●) the presence of (A) 5 mM ASN, (B) 5 mM GSNO and (C) 2 mM NOC5/7 and incubated at 37°C for 7 hours. Bacteria were serially diluted in PBS and quantified by plating onto LB agar by the method of Miles and Misra and grown at 37°C statically overnight. Data points represent the mean of three independent repeats performed in triplicate, error bars represent standard error.

3.3.2 A *copA/golT* double mutant of S. Typhimurium has reduced tolerance to reactive nitrogen species

Although copper does not increase the potency of reactive nitrogen species to S. Typhimurium, reactive nitrogen species are known to liberate copper from metalloproteins (Gold *et al.* 2008). Hence, copper homeostatic mutants of S. Typhimurium may have reduced tolerance to reactive nitrogen species. To test this hypothesis the tolerance of copper homeostasis mutants to reactive nitrogen species was investigated. If copper is being liberated from bound ligands within S. Typhimurium, reduced growth of *a* copper homeostasis would be expected.

SL1344, *ΔcopA/ΔgolT*, *ΔcopA/ΔgolT/Δhmp* and *Δhmp* were grown in the presence of LB, ASN, GSNO and NOC5/7 (figures 3.10). A S. Typhimurium 14028 Δ*hmp* mutant, which encodes a flavohaemoglobin that detoxifies reactive nitrogen species, was received from Professor Roberts Poole's research group and transferred by P22 phage transduction intoSL1344 and *ΔcopA/ΔgolT* to obtain a *ΔcopA/ΔgolT/Δhmp* triple mutant. In the presence of nitric oxide under aerobic conditions a *hmp* mutant should have inhibited growth and is utilised in these experiments as a positive control to indicate that a nitric oxide stress is present. Also to assay the presence of nitrite within the culture an equal volume of culture and Griess reagent were added together, which upon the presence of nitrite produces a colorimetric change. Griess reagent revealed the presence of nitrite within all cultures when supplemented with a reactive nitrogen species donor (data not shown) indicating that a mixture of reactive nitrogen species are produced during the experiment. The *hmp* mutant grew poorly under the stress of all of the reactive nitrogen species generators confirming reactive nitrogen species are produced by ASN, GSNO and NOC5/7. The *copA/golT* double mutant had significantly reduced growth in comparison to SL1344 for all reactive nitrogen species generators ($p < 0.05$). The addition of a *hmp* mutation to a *copA/golT* double mutant gave an additive effect, with *ΔcopA/ΔgolT/Δhmp* having reduced reactive nitrogen species tolerance than *ΔcopA/ΔgolT*. This suggests that reactive nitrogen species may be liberating bound copper within S. Typhimurium which SL1344 has the necessary copper detoxification mechanisms to remove copper stress that a *copA/golT* double mutant cannot. Alternatively copper export by CopA and GolT is part of the defence system against reactive nitrogen species. It is known that Cu^{2+} can oxidise nitric oxide forming a nitrosonium cation, which can react with thiol amines to form S-nitrothiols; alternatively Cu^{+} can reduce S-nitrothiols to release nitric oxide (Stamler *et al.* 1992, Singh *et al.* 1996). Thus, copper can potentially drive reactive nitrogen species cycling between nitric oxide and nitrosonium ions, increasing the potency of reactive nitrogen species.

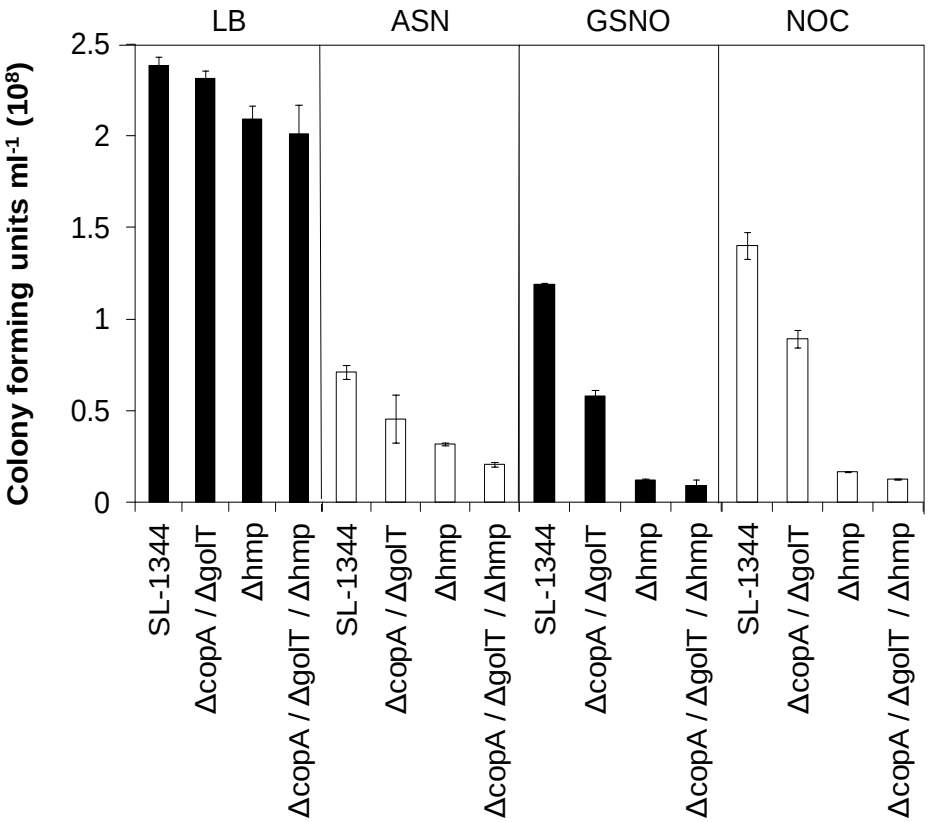


Figure 3.10 *ΔcopA/ΔgolT* has reduced tolerance to reactive nitrogen species

Overnight cultures of SL1344, *ΔcopA/ΔgolT*, *Δhmp* and *ΔcopA/ΔgolT/Δhmp* were diluted 1/1000 into fresh LB (ASN LB buffered at pH 5.5) and supplemented with various concentrations reactive nitrogen species generators: ASN, GSNO and NOC5/7 5/7 and incubated at 37°C for 7 hours. Bacteria were serially diluted in PBS and quantified by plating onto LB agar by the method of Miles and Misra and grown at 37°C statically overnight. Data points represent the mean of three independent repeats performed in triplicate, error bars represent standard error.

To confirm the reduced growth of a *copA/golT* double mutant to that of SL1344 in the presence of reactive nitrogen species a growth curve assay was performed in the presence of ASN (figure 3.11). Overnight cultures of SL1344, *ΔcopA/ΔgolT* and *Δhmp* were diluted 1/1000 into fresh LB in the presence and absence of 5 mM ASN (figure 3.11). SL1344, *ΔcopA/ΔgolT* and *Δhmp* all grew to a similar OD_{600} value when grown in LB, reaching stationary phase by 7 hours. In the presence of ASN growth of *Δhmp* and *ΔcopA/ΔgolT* were inhibited. SL1344 reached a final OD_{600} of 2.12 (±0.05), *ΔcopA/ΔgolT* to an OD_{600} of 1.85 (±0.03) and *Δhmp* to an OD_{600} of 1.18 (±0.20). This confirmed that in the presence of reactive nitrogen species *ΔcopA/ΔgolT* has reduced growth compared to SL1344.

Both CopA and GolT are functionally redundant capable of replacing each other when either is absent or not functional. A copper sensitive phenotype is only seen when a double mutation of both *copA* and *golT* is present. To check whether *copA* or *golT* single mutants exhibit reduced tolerance to reactive nitrogen species, tolerance assays were performed using all three reactive nitrogen species generators as previously described (figure 3.12). No difference was seen between SL1344 *ΔcopA* and *ΔgolT* for all reactive nitrogen species generators whilst *ΔcopA/ΔgolT* consistently grew significantly lower. This supported the hypothesis that reactive nitrogen species is liberating copper within S. Typhimurium as a *ΔcopA* or *ΔgolT* does not have any difference in copper homeostasis to SL1344.

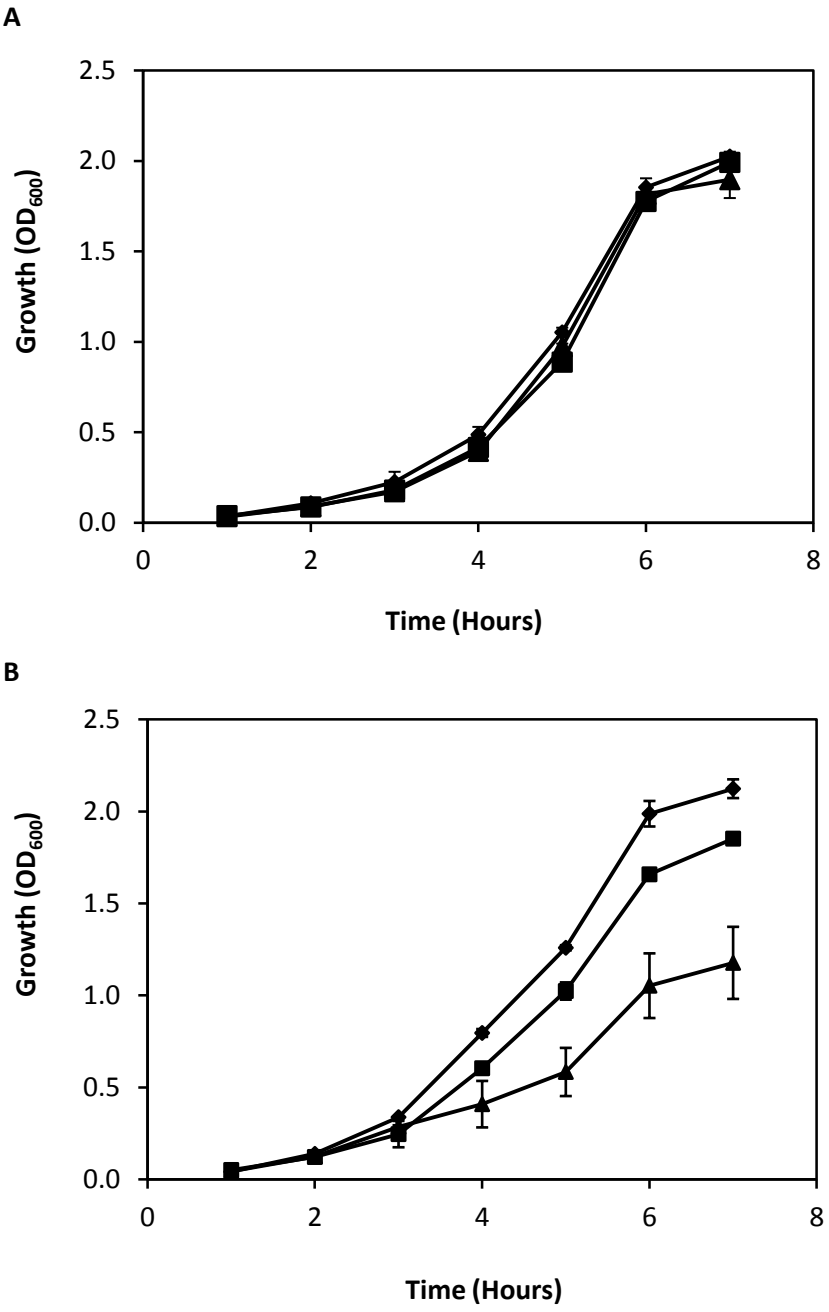
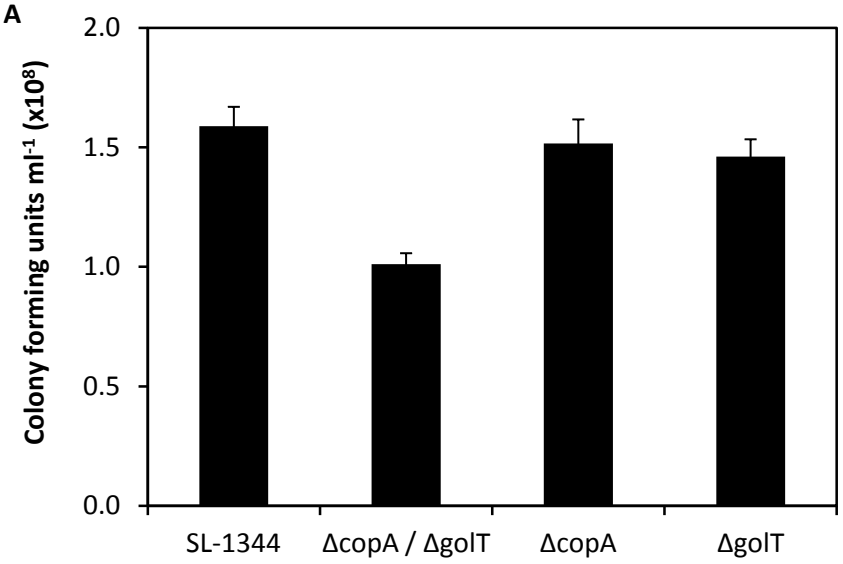


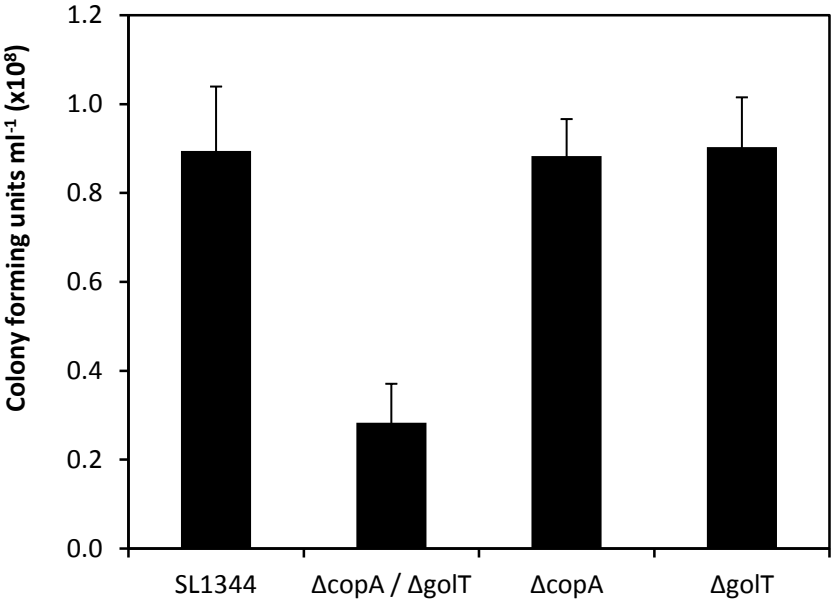
Figure 3.11 *ΔcopA/ΔgolT* has reduced growth in the presence of ASN

OD_{600} readings for overnight cultures of SL1344 (♦), *ΔcopA/ΔgolT* (■) *and Δhmp* (▲) diluted 1/100 into fresh LB media buffered at pH 5.5 (A) or supplemented with 5 mM ASN (B) and incubated at 37°C 200 rpm for 7 hours. Data points represent the mean of one repeat performed in triplicate, error bars represent standard error.

A


Colony forming units ml-1 (x108)
2.0
1.5
1.0
0.5
0.0
SL-1344
ΔcopA / ΔgolT
ΔcopA
ΔgolT

B


Colony forming units ml-1 (x108)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
SL1344
ΔcopA / ΔgolT
ΔcopA
ΔgolT

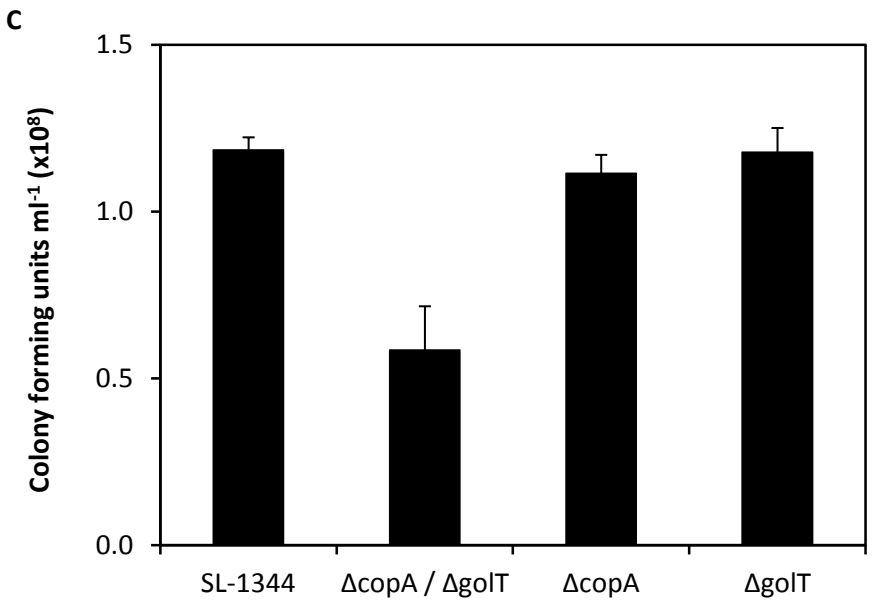


Figure 3.12 An S. Typhimurium *copA/golT* double mutant has reduced growth in the presence of ASN, GSNO and NOC5/7 5/7, but not *copA* or *golT* single mutant

Overnight cultures of SL1344, *ΔcopA/ΔgolT*, *ΔcopA* and *ΔgolT* were diluted 1/1000 into fresh LB media in the presence of: (A) 5 mM ASN (LB buffered to pH 5.5), (B) 5 mM GSNO and (C) 2 mM NOC5/7 then incubated at 37°C for 7 hours. Bacteria were serially diluted in PBS and quantified by plating onto LB agar by the method of Miles and Misra and grown at 37°C statically overnight. The following day colonies were counted and converted to cfu ml^{-1}. Data points represent the mean of three independent repeats performed in triplicate, error bars represent standard error.

3.3.3 GSNO and NOC5/7 5/7 induce the expression of *copA* or *golT* in S. Typhimurium

If reactive nitrogen species are liberating copper from metalloproteins within S. Typhimurium then it is anticipated that this would be detected by the copper-responsive transcription regulator CueR and GolS leading to elevated expression of the *cue* and *gol* copper resistance systems. The lack of copper export ability in a *copA/golT* double mutant would lead to increased sensitivity to reactive nitrogen species. Expression from the *copA* and *golT* promoters can be used to monitor cytosolic copper levels within S. Typhimurium (Osman *et al.* 2010) and hence identify if reactive nitrogen species are liberating complexed copper (figures 3.13 and 3.14). The expression of *copA* and *golT* were monitored by β-galactosidase assays in the presence of GSNO and NOC5/7. If copper is liberated by the reactive nitrogen species generators then expression of *copA* and *golT* would be expected to be seen. Constructs containing the promoter region of *copA* and *golT* had been cloned into the multicopy plasmid pRS415 containing a *lacZ* gene encoding a β-galactosidase enzyme. The constructs were made previously within the laboratory group and were sequenced to confirm the correct identity (Osman *et al.* 2010). β-galactosidase assays were attempted with ASN but the buffered acidic pH of 5.5 activated expression of *lacZ* and gave false positive results (data not shown), consequently *copA* and *golT* expression could not be monitored for ASN. A slight induction was seen for either *copA* or *golT* when exposed to either GSNO or NOC5/7. A positive control of 100 μM $CuSO_4$ was used and gave an 8 fold induction for expression of both *copA* and *golT* confirming the promoters are responsive to copper (not shown). This hints that copper may be liberated from bound complexes by GSNO and NOC5/7.

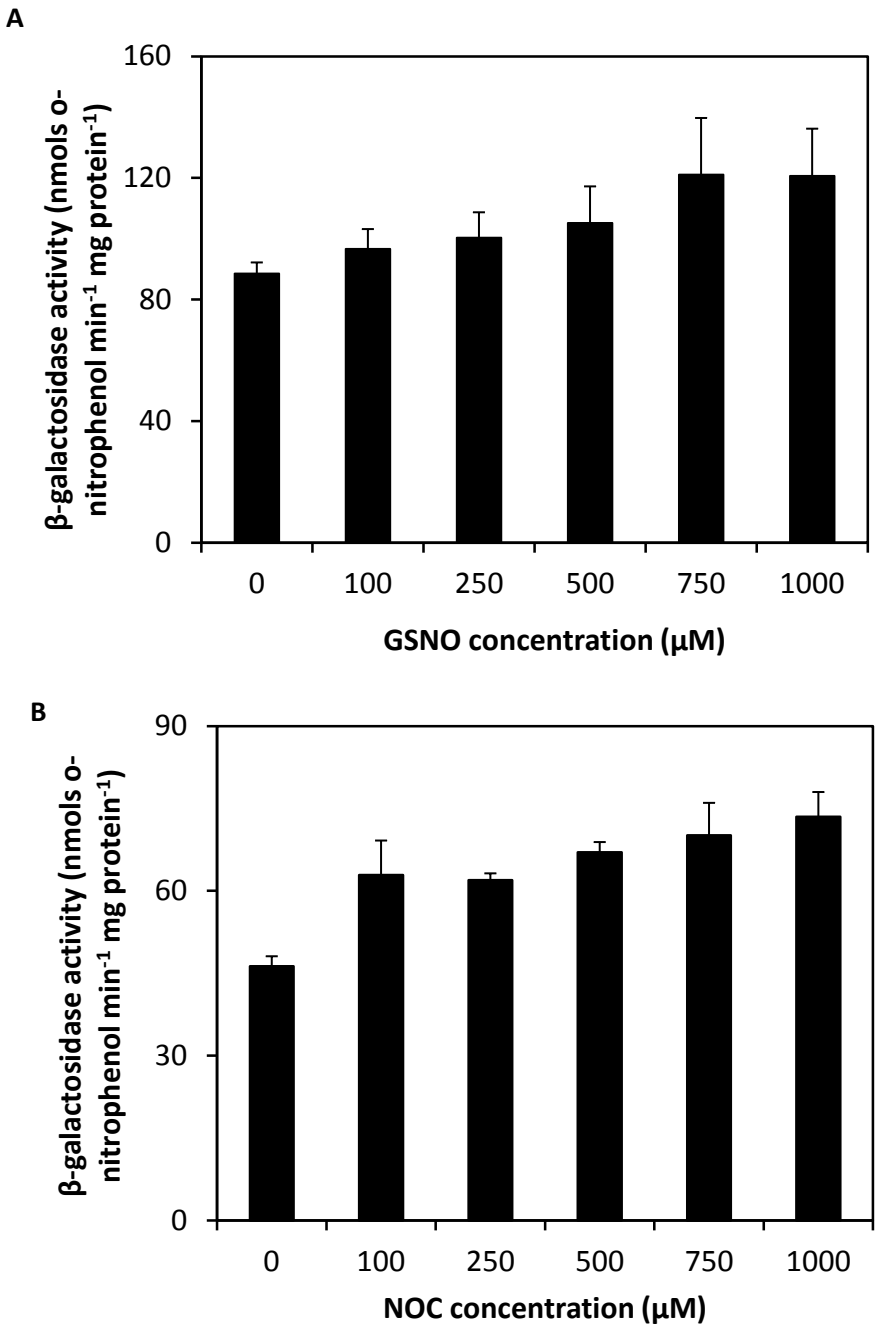


Figure 3.13 GSNO and NOC5/7 induce expression of *copA* in SL1344

β-galactosidase activity of SL1344 containing pRS*copA* grown in the presence/absence of (A) GSNO and (B) NOC5/7. An overnight culture of SL1344 containing pRS*copA* was diluted 1/100 into fresh LB containing ampicillin and incubated at 37°C 200 rpm until an OD_{600} 0.3 was reached and used in β-galactosidase assays. Data points represent the mean of three independent repeats performed in triplicate, error bars represent standard error.

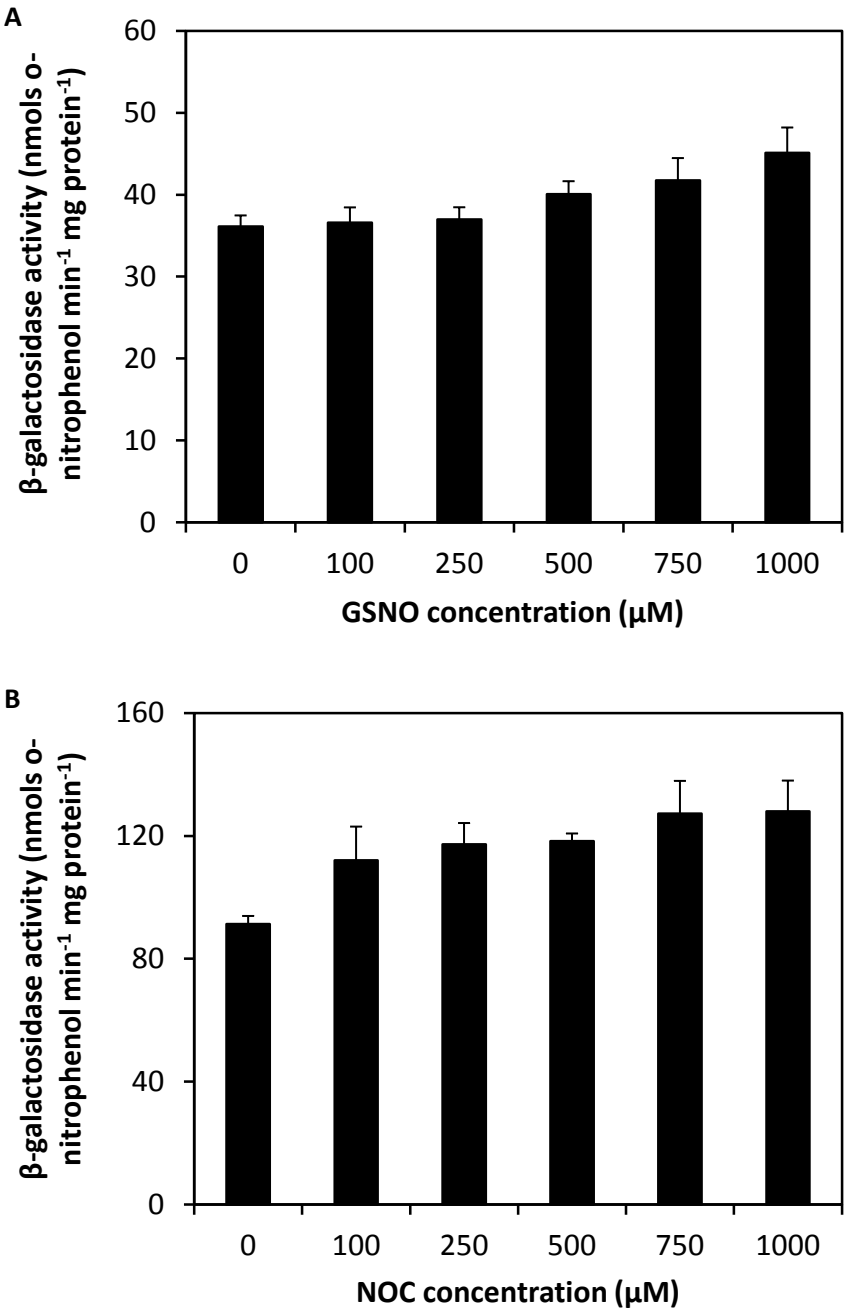


Figure 3.14 GSNO and NOC5/7 induce expression of *golT* in SL1344

β-galactosidase activity of SL1344 containing pRS*golT* grown in the presence/absence of (A) GSNO and (B) NOC5/7. An overnight culture of SL1344 containing pRS*golT* was diluted 1/100 into fresh LB containing ampicillin and incubated at 37°C 200 rpm until an OD_{600} 0.3 was reached and used in β-galactosidase assays. Data points represent the mean of three independent repeats performed in triplicate, error bars represent standard error.

The slight induction of *copA* and *golT* by both GSNO (1.36 + 1.25) and NOC5/7 (1.59 + 1.40) suggests copper could be liberated by reactive nitrogen species. This observation suggests a role for CopA and GolT in reactive nitrogen species defence. The induction of *copA* and *golT* supports previous data (figure 3.10) that a *copA/golT* double mutant has decreased replication in the presence of ASN, GSNO and NOC.

3.4 A *copA/golT* double mutant has no difference in peroxynitrite killing to SL1344

Having investigated the roles of reactive oxygen species and reactive nitrogen species the final reactive species peroxynitrite was investigated. Peroxynitrite is the combination of nitric oxide and superoxide as shown below.

$O_2^- + NO^\cdot \rightarrow ONOO^-$

Peroxynitrite has both oxidising and nitrosation effects on bacterial pathogens. Tetramethylammonium peroxynitrite was purchased from Sigma-Aldrich that readily decomposes releasing peroxynitrite. To determine the effective concentrations of peroxynitrite to kill S. Typhimurium, titrations were initially performed with SL1344 (figure 3.15). At a concentration of 2 mM peroxynitrite an SL1344 viable count of $1.69x10^7$ bacteria survived in comparison to $3.67x10^7$ incubated in LB broth without peroxynitrite. Subsequent experiments performed were therefore performed with 2 mM peroxynitrite concentration.

Peroxynitrite killing of S. Typhimurium in the presence of copper was investigated to identify if copper could influence peroxynitrite potency and if copper homeostasis mutants have reduced tolerance to peroxynitrite (figure 3.16). No difference in peroxynitrite tolerance was seen in the presence or absence of copper. This indicates that copper does not increase the potency of peroxynitrite to S. Typhimurium. No difference of the copper homeostasis mutant, *hmp* mutant or SL1344 to peroxynitrite was observed in the presence or absence of copper. This identified that peroxynitrite does not give a strong reactive nitrogen species response due to *Δhmp* and *ΔcopA/ΔgolT/Δhmp* having a similar viable count to that of SL1344 and *ΔcopA/ΔgolT*. As seen with reactive oxygen species experiments, cellular copper levels do not affect peroxynitrite killing due to *ΔcopA/ΔgolT* having a similar survival to SL1344. These findings suggest that peroxynitrite toxicity is not influenced by the presence of copper.

An alternate method to produce peroxynitrite was performed, superoxide and nitric oxide can form peroxynitrite, as shown below.

$NO + O_2^- \rightarrow ONOO^-$

Hence, further analysis the tolerance of SL1344 and copper homeostatic mutants to peroxynitrite was performed by incubating S. Typhimurium in the presence of 50 µM hydrogen peroxide and 5 mM ASN (figure 3.17). The combined stress of hydrogen peroxide and ASN yielded no difference in the survival between copper homeostasis and reactive nitrogen species homeostasis mutants compared to SL1344. The addition of ASN to hydrogen peroxide gave no difference in the survival of the various strains compared to hydrogen peroxide exposure alone. No phenotype was seen as previously of a *copA/golT* double mutant and *hmp* mutant, when exposed to ASN alone (figure 3.10). Indeed, the sensitivity to reactive nitrogen species is a difference in growth between the strains, whereas the addition of hydrogen peroxide induces killing of S. Typhimurium, preventing a reactive nitrogen species mediated phenotype.

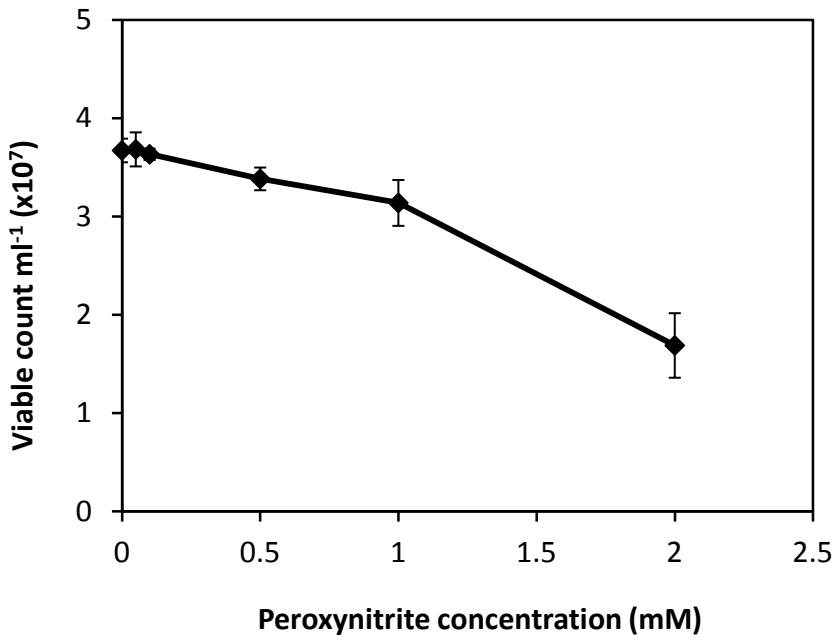


Figure 3.15 Tolerance of SL1344 to increasing concentrations of peroxynitrite

An overnight culture of SL1344 was diluted 1/100 into fresh LB and incubated at 37°C for 2 hours with varying concentrations of peroxynitrite. Bacteria were serially diluted in PBS and quantified by plating onto LB agar by the method of Miles and Misra and grown at 37°C statically overnight. Data points represent the mean of one repeat performed in triplicate, error bars represent standard error.

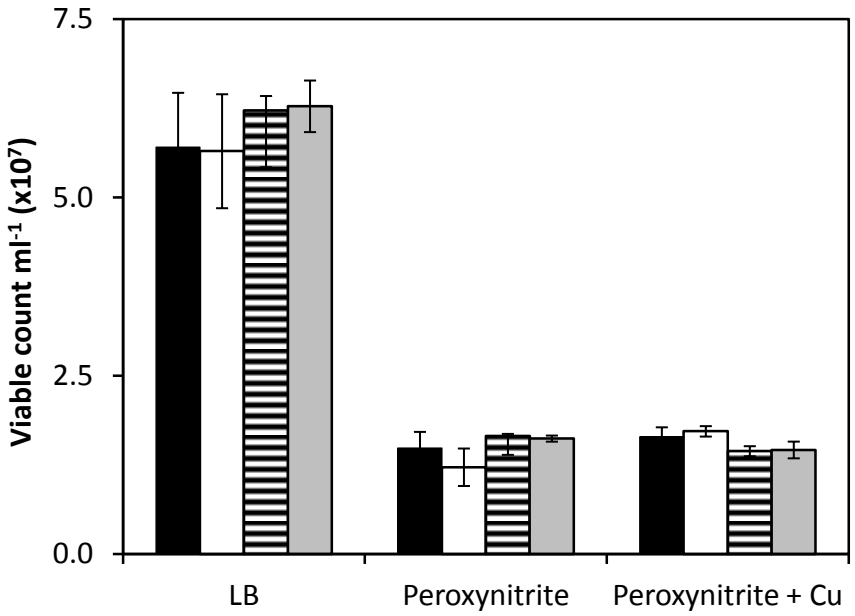


Figure 3.16 Copper does not increase peroxynitrite toxicity toward S. Typhimurium, *ΔcopA/ΔgolT* and *Δhmp* show similar tolerance to peroxynitrite as SL1344

Overnight cultures of SL1344 (black bars), *ΔcopA/ΔgolT* (white bars), *Δhmp* (striped bars) and *ΔcopA/ΔgolT/Δhmp* (grey bars) were diluted 1/100 into fresh LB and incubated at 37°C for 2 hours with 2 mM peroxynitrite with and without 0.25 mM $CuSO_4$. Bacteria were serially diluted in PBS and quantified by plating onto LB agar by the method of Miles and Misra and grown at 37°C statically overnight. Data points represent the mean of three independent repeats performed in triplicate, error bars represent standard error.

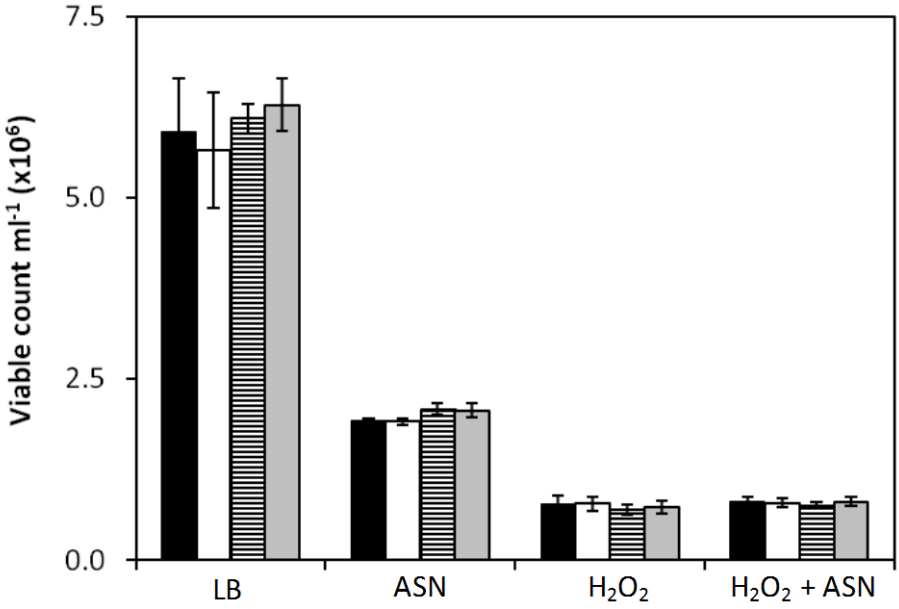


Figure 3.17 S. Typhimurium *ΔcopA/ΔgolT* or *Δhmp* mutants have similar tolerance to SL1344 in the combined stress of H_2O_2 and ASN

Overnight cultures of SL1344 (black bars), *ΔcopA/ΔgolT* (white bars), *Δhmp* (striped bars) and *ΔcopA/ΔgolT/Δhmp* (grey bars) were diluted 1/100 into fresh LB and incubated at 37°C for 2 hours with 5 mM ASN and 50 µM H_2O_2 as indicated. Bacteria were serially diluted in PBS and quantified by plating onto LB agar by the method of Miles and Misra and grown at 37°C statically overnight. Data points represent the mean of three independent repeats performed in triplicate, error bars represent standard error.

3.5 Discussion

3.5.1 Free copper enhances the toxicity of hydrogen peroxide towards S. Typhimurium

Data obtained confirmed previous reports that copper is able to increase the toxicity of hydrogen peroxide towards bacteria through Fenton chemistry (Aust *et al.* 1985, White *et al.* 2009). The addition of 1 mM copper sulphate to 50 µM hydrogen peroxide gave an increase in killing of one log than addition of hydrogen peroxide alone (figure 3.1). Copper is capable of undergoing redox reactions by accepting and donating electrons to oxygen based molecules resulting in the formation of hydroxyl radicals that can damage DNA, protein and lipids. The presence of copper increases the conversion of hydrogen peroxide into hydroxyl radicals. No difference in susceptibility to hydrogen peroxide mediated killing was observed between SL1344 and *ΔcopA/ΔgolT*. *ΔcopA/ΔgolT* has been identified to accumulate approximately 25 fold more copper than SL1344 when grown in media supplemented with copper (Osman *et al.* 2010). Despite *ΔcopA/ΔgolT* having significantly greater internal copper levels than SL1344, *ΔcopA/ΔgolT* must therefore be capable of complexing its internal copper, such that it is unable to partake within redox reactions that generate hydroxyl radicals.

The ability of copper to catalyse Fenton chemistry significant increases reactive oxygen species damage. White *et al.* (2009) identified that physiological concentrations of hydrogen peroxide and copper present within a macrophage phagosome are toxic to *E. coli*, and mutation of the copper exporter, *copA*, had increased killing. This suggests that copper homeostasis is important for *E. coli* intracellular infection to avoid Fenton chemistry. Although, *Salmonella* copper homeostatic mutant strains do not exhibit decreased tolerance to reactive oxygen species as seen within *E. coli*.

3.5.2 Copper does not influence the potency of paraquat and xanthine oxidase towards S. Typhimurium

Paraquat and xanthine oxidase were investigated as alternate reactive oxygen species generators to hydrogen peroxide. Paraquat generates superoxide within S. Typhimurium and xanthine oxidase produces hydrogen peroxide and superoxide outside of S. Typhimurium (Kelley *et al.* 2010). The addition of copper to either reactive oxygen species generator did not give increased killing of S. Typhimurium. Paraquat generates superoxide cycling between an aqueous and radical state reducing oxygen upon returning to its aqueous state

as shown in section 3.1.3. Copper could potentially aid the conversion of paraquat into its radical state by donating electrons that paraquat typically receives from NADPH (Hassan and Fridovich 1979). However, as shown by the inability of copper to catalyse Fenton chemistry within *Salmonella*, internalised copper is unable to participate in chemical reactions. Therefore, it is expected that copper is unable to increase the potency of paraquat. It has been reported that copper potentiates paraquat killing within *E. coli* (Kohen and Chevion 1985). S. Typhimurium and *E. coli* are closely related organisms but have different copper and reactive oxygen species detoxification systems. S. Typhimurium possess four superoxide dismutase enzymes; two Cu, Zn Sod's within the periplasm and an Fe and Mn Sod enzyme in the cytosol. *E. coli* also has an Fe and Mn Sod within the cytosol but only one Cu, Zn Sod within the periplasm. The lack of another periplasmic Sod may leave *E. coli* more susceptible to superoxide toxicity than S. Typhimurium. As suggested in White *et al.* (2009) internalised copper within *E. coli* is able to participate in Fenton chemistry and could also interact with paraquat increasing its potency, which seems not to occur in *Salmonella*. No difference in paraquat killing was seen across *Salmonella* copper homeostasis mutants (figure 3.4). Having previously identified that copper does not potentiate paraquat toxicity in S. Typhimurium it was expected that copper homeostasis mutants have the same level of killing as SL1344. This is consistent with previous reports that S. Typhimurium copper homeostasis mutants are not more susceptible to reactive oxygen species (Osman *et al.* 2010, Vollmecke *et al.* 2012).

Copper also did not increase the toxicity of reactive oxygen species produced by xanthine oxidase towards S. Typhimurium. Xanthine oxidase converts hypoxanthine and xanthine into hydrogen peroxide and superoxide, as shown in section 3.1.4. Copper is known to catalyse the formation of hydroxyl radicals by Fenton chemistry potentiating the toxicity of hydrogen peroxide and copper as seen in figure 3.1. Xanthine oxidase produces low levels of hydrogen peroxide and superoxide approximately 1.6 µM and 0.4 µM per 2 min, respectively (McCord and Fridovich 1969). The addition of copper did not give increased killing by xanthine oxidase possibly due to only a low concentration of hydrogen peroxide present at any given time during the experiment. The smaller quantity of hydrogen peroxide may receive an adequate source of electrons to promote Fenton chemistry from NADPH and leakage from the electron transport chain and therefore not require copper to catalyse Fenton chemistry. Initially when exposed to xanthine oxidase and hypoxanthine S. Typhimurium is exposed to approximately 1.6 µM hydrogen peroxide within the first 2 mins of the experiment; whereas upon addition of hydrogen peroxide S. Typhimurium is exposed to 100 µM hydrogen peroxide. Electron leakage from the electron transport chain may supply enough electrons to catalyse Fenton chemistry of up to 2 µM hydrogen peroxide per

min, but this would not be sufficient to catalyse 100 µM hydrogen peroxide. The addition of copper provides an alternate reducing agent that converts a larger proportion of the 100 µM hydrogen peroxide into hydroxyl radicals, which is potentially why the addition of copper to hydrogen peroxide increasing its potency but addition of copper to xanthine oxidase has no effect.

3.5.3 A catalase mutant has increased sensitivity to hydrogen peroxide but not paraquat and xanthine oxidase

Reactive oxygen species tolerance of a *ΔkatG*, catalase mutant was investigated. *ΔkatG* had reduced tolerance to hydrogen peroxide, having an approximate 2.5 fold decrease in survival at 0.1 mM hydrogen peroxide compared to SL1344. *ΔkatG* had no difference to SL1344 for paraquat or xanthine oxidase generated reactive oxygen species tolerance. Catalase converts hydrogen peroxide into water and oxygen and has a remarkably high rate of reaction and does not become saturated in the presence of hydrogen peroxide (Lledias *et al.* 1998). The sensitivity of a *katG* mutant confirms the importance of catalase for detoxifying hydrogen peroxide within S. Typhimurium (section 3.1.5). KatG is one of three catalase enzymes that degrade hydrogen peroxide within *Salmonella*; KatE and KatN are the two other catalase enzymes that are located in the cytosol. Other enzymes such as glutathione peroxidase (ButE) and two alkyl hydroperoxide reductases (AphC and TsaA) are capable of detoxifying hydrogen peroxide (Morgan *et al.* 1986, Hebrard *et al.* 2009). Hebrard *et al.* (2009) reported to only see sensitivity to hydrogen peroxide in a S. Typhimurium *katE/katG/katN* triple mutant. On these findings we propose that catalase enzymes do not functionally replace an individual catalase mutant, and that removal of each catalase may lower the overall tolerance to hydrogen peroxide incrementally, such that a strain such as *ΔkatE/ΔkatG/ΔkatN* is highly susceptible to hydrogen peroxide mediated killing. KatG is the only known periplasmic hydrogen peroxide detoxification enzyme with: KatE, KatN, AphC, TsaA and BtuE located within the cytosol. The decrease in tolerance to hydrogen peroxide of a *katG* mutant compared to SL1344 may be due to reactive oxygen species damage to the outer membrane that is typically prevented by KatG activity.

Paraquat generates superoxide which catalase cannot detoxify but is detoxified by superoxide dismutase enzymes. The presence of superoxide would therefore result in an increased presence of hydrogen peroxide converted by superoxide dismutase enzymes. No difference in *ΔkatG* and SL1344 paraquat tolerance indicates that remaining hydrogen

peroxide detoxifying enzymes are capable of removing superoxide dismutase converted hydrogen peroxide. As previously stated, xanthine oxidase produces a low level of hydrogen peroxide and superoxide continuously throughout the experiment. This level is not a sufficient concentration to have an antimicrobial effect on a *katG* mutant due to the presence of the alternate hydrogen peroxide and superoxide detoxifying enzymes.

A *katG* mutation was investigated in combination with a *copA/golT* double mutation. Catalase inhibits Fenton chemistry by removing hydrogen peroxide, the removal of KatG will result in a greater proportion of hydrogen peroxide reaching the cytosol, which in a *copA/golT* double mutant has a high copper quota. No difference in reactive oxygen species tolerance was observed between: *ΔkatG*, *ΔcopA/ΔgolT/ΔkatG* and *ΔsodC$_I$/ΔsodC$_{II}$/ΔkatG*. *ΔcopA/ΔgolT/ΔkatG* had no difference in hydrogen peroxide tolerance to *ΔkatG*, confirming previous data (section 3.2.4) that internalised copper does not interact with hydrogen peroxide. This study confirmed previous work on S. Typhimurium that identified a *sodC$_I$/sodC$_{II}$* double mutant does not have increased sensitivity to reactive oxygen species, with *ΔsodC$_I$/ΔsodC$_{II}$/ΔkatG* and *ΔkatG* having a similar hydrogen peroxide tolerance (Osman *et al.* 2010, Craig and Slauch 2009). *Salmonella* contains two cytoplasmic superoxide dismutase enzymes, SodA and SodB, which are likely detoxifying superoxide produced by paraquat and xanthine oxidase. An overview of the reactive oxygen species detoxification system within S. Typhimurium is shown in figure 3.18.

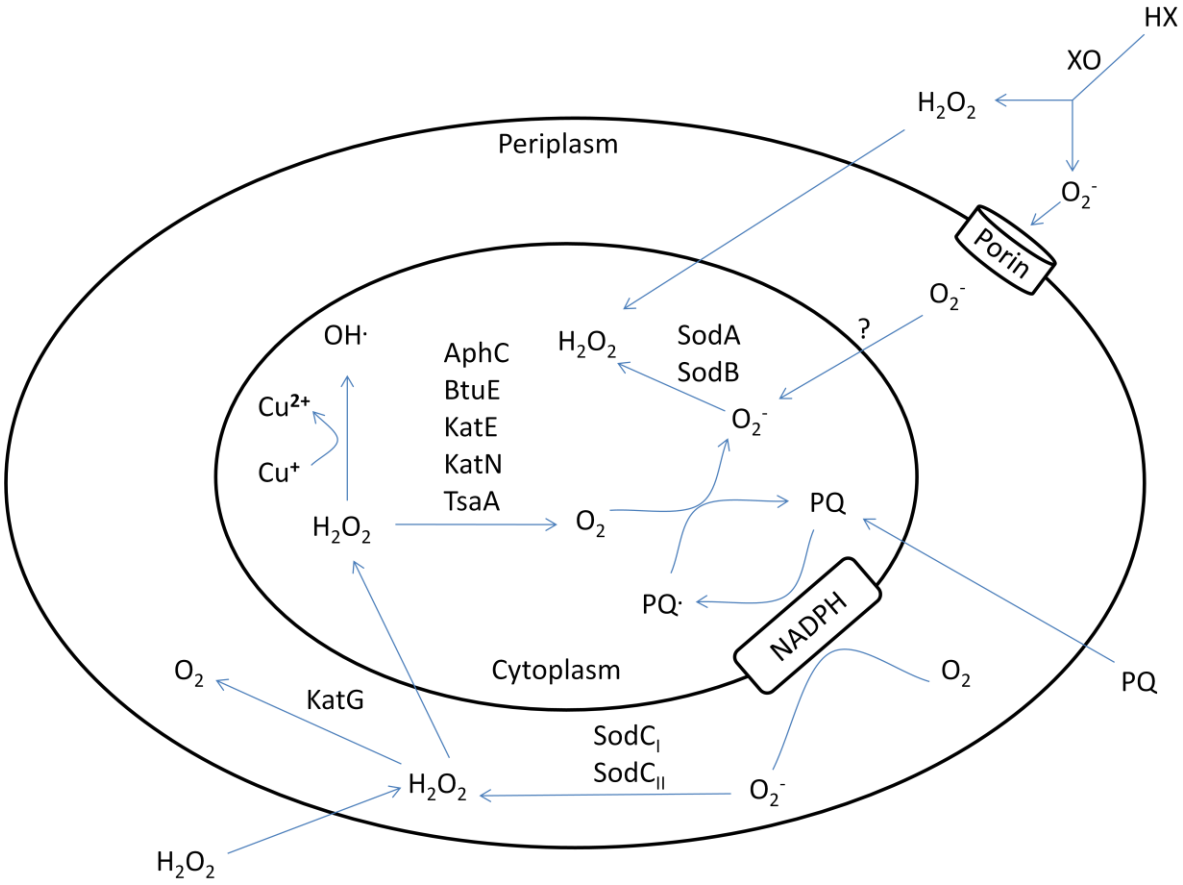


Figure 3.18 Reactive oxygen species detoxification mechanisms of S. Typhimurium

Hydrogen peroxide (H_2O_2) present in the external environment or formed by the breakdown of hypoxanthine (HX) by xanthine oxidase (XO) and can passively diffuse into the periplasm and cytosol. In the periplasm hydrogen peroxide can be converted into oxygen (O_2) and water (H_2O) by the periplasmic catalase, KatG. Cytoplasmic hydrogen peroxide can be converted into oxygen and water by several enzymes: two catalases KatN and KatE, two alkyl hydroperoxide reductases AphC and TsaA and glutathione peroxidase ButE. Alternatively, hydrogen peroxide can undergo Fenton chemistry in the presence of a redox capable metal such as copper, generating highly toxic hydroxyl radicals ($OH^{\cdot}$). Superoxide is generated extracellularly by the breakdown of hypoxanthine by xanthine oxidase and enters the periplasm through porins present in the outer membrane. In the periplasm superoxide can be detoxified by two superoxide dismutase enzymes, $SodC_I$ and $SodC_{II}$ into oxygen. Superoxide can be formed from the leakage of electrons from NADPH and is estimated to account for 1-3% of electron transport by NADPH. It is currently unknown how superoxide enters the cytosol. Superoxide can also be formed within the cytosol by the cycling of paraquat (PQ), which can freely diffuse into the cytoplasm from the external environment. Paraquat receives electrons from NADPH and forms a paraquat radical ($PQ^{\cdot}$) that reforms paraquat by reducing oxygen into superoxide. Superoxide is detoxified in the cytoplasm by two superoxide dismutase enzymes, SodA and SodB into hydrogen peroxide.

3.5.4 Copper does not potentiate the toxicity of reactive nitrogen species towards S. Typhimurium

Gold *et al.* (2008) identified that reactive nitrogen species can liberate copper from a bound metallothionine within *M. tuberculosis* which led to the question; could reactive nitrogen species liberate copper within S. Typhimurium? Three reactive nitrogen species generators were selected to give a range of reactive nitrogen species. ASN gives a mixture of reactive nitrogen species, GSNO is a nitrosylating agent that directly adds a nitryl group to amines and NOC5/7 releases nitric oxide at regular timings. Reactive nitrogen species are known to target multiple macromolecules within a bacterium, including similar targets to copper such as iron-sulphur clusters (Copper 1999).

No difference was seen in the tolerance of SL1344 to copper in the presence and absence of sub-lethal concentrations of reactive nitrogen species ASN, GSNO and NOC5/7. This indicates that reactive nitrogen species and copper do not exaggerate the toxicity of each other as seen within Fenton chemistry between hydrogen peroxide and copper. It has been identified that copper is capable of increasing the rate of GSNO mediated nitrosylation (Gorren *et al.* 1996). Although, GSNO is not a stable compound, it has a half life of approximately 15 mins which is reduced by >4°C, light and the presence of metals. Therefore the addition of copper to GSNO may have had little impact increasing the rate of GSNO mediated nitrosylation.

Djoko *et al.* (2012) identified that a *N. gonorrhoeae copA* mutant has increased susceptibility to reactive nitrogen species in the presence of copper. It is known that copper can drive reactive nitrogen species cycling (Stamler *et al.* 1992, Singh *et al.* 1996). *N. gonorrhoeae* and *Salmonella* copper homeostasis systems differ, *N. gonorrhoeae* does not encode a CueR homologue, with an affinity for less than one free copper atom within a cell (Changela *et al.* 2003, Djoko *et al.* 2012). This suggests that *N. gonorrhoeae* does not have as tight regulation of internal copper levels as *Salmonella*, and is susceptible to reactive nitrogen species cycling.

3.5.5 A *copA/golT* double mutant has decreased growth in the presence of reactive nitrogen species

Osman *et al.* (2010) reported that a *copA/golT* double mutant accumulates significantly greater copper levels than SL1344. The reactive nitrogen species tolerance of *ΔcopA/ΔgolT* was investigated due to having increased levels of internalised copper, if reactive nitrogen

species can liberate copper within S. Typhimurium, *ΔcopA/ΔgolT* has significantly greater internalised copper that could be released. A *copA/golT* double mutant had decreased growth in the presence of all reactive nitrogen species generators. *ΔcopA/ΔgolT* had a reduction in viable colonies of 1.75, 2.30 and 1.65 fold for ASN, GSNO and NOC5/7, respectively compared to SL1344. A *hmp* mutant was utilised as a positive control due to its sensitivity to nitric oxide and nitrite (Stevanin *et al.* 2002, Gilberthorpe *et al.* 2007). Hmp is the only major mechanism of aerobic reactive nitrogen species detoxification and the reduced growth of the *hmp* mutant in the presence of each reactive nitrogen species confirmed the production of reactive nitrogen species (figure 3.10, Stevanin *et al.* 2002). To further investigate the reduced growth of the *copA/golT* double mutant, single mutants for *copA* and *golT* were also grown in the presence of reactive nitrogen species but did not have reduced growth as seen for a *copA*/ *golT* double mutant (figure 3.12). This suggested that the reduced growth was a copper mediated phenotype. A *copA/golT* double mutant is highly sensitive to copper whereas a *copA* or *golT* single mutant has similar copper tolerance to that of SL1344 (Osman *et al.* 2010). SL1344, *ΔcopA/ΔgolT, ΔcopA* and *ΔgolT* were incubated in the presence of ASN, GSNO and NOC5/7 with the addition of a sub-lethal $CuSO_4$ (10 μM), but no difference was seen to incubation with reactive nitrogen species donors in the absence of copper (figure 3.12). These data suggests that copper may not potentiate reactive nitrogen species cycling as seen within *N. gonorrhoeae* (Djoko *et al.* 2012).

To analyse if copper was being liberated by reactive nitrogen species *copA* and *golT* expression was monitored through β-galactosidase assays. A slight induction of both *copA* and *golT* of 1.36 and 1.25 fold increase when exposed to GSNO and 1.59 and 1.40 fold increase when exposed to NOC5/7. The induction of *copA* and *golT* by GSNO and NOC5/7 suggests that copper can be released from a complexed state within *Salmonella*. This observation has been previously reported within *M. tuberculosis* by Gold *et al.* (2008). The slight induction of *copA* and *golT* observed is potentially due to SL1344 having low intracellular copper levels, hence reactive nitrogen species have a small chance to interaction with a copper complex. By monitoring *copA* and *golT* expression within a *copA/golT* double mutant a greater induction may be seen from the increased potential for reactive nitrogen species to encounter complexed copper due to an greater intracellular copper load (Osman *et al.* 2010).

Expression of *copA* within *N. gonorrhoeae* is independent of copper levels, and regulated by the MerR regulator NmlR, which has been reported to provide resistance against nitrosative stress within *N. gonorrhoeae* (McEwan *et al.* 2011, Djoko *et al.* 2012). Djoko *et al.* (2012)

highlighted that tolerance to reactive nitrogen species and copper within *N. gonorrhoeae* are closely linked. The reduced growth of a S. Typhimurium *copA/golT* double mutant exposed to reactive nitrogen species and the induction of *copA* and *golT* by GSNO and NOC5/7 highlights a potential link between copper and reactive nitrogen species tolerance within S. Typhimurium.

3.5.6 Copper homeostasis mutants and SL1344 have the same tolerance to peroxynitrite

Peroxynitrite is a potent form of reactive species formed by the combination of superoxide and nitric oxide. Peroxynitrite has been identified to generate both reactive oxygen species and reactive nitrogen species mediated damage to bacteria (Bonini *et al.* 1999). Here, copper was found to not potentiate peroxynitrite killing of S. Typhimurium. This is consistent with there being no reported mechanism of copper interacting with peroxynitrite. Peroxynitrite is readily capable of acting as a nucleophile and does not require undergoing any redox reactions prior to exhibiting antimicrobial effects. Therefore it was of no surprise to see the addition of copper to peroxynitrite did not affect S. Typhimurium survival. No difference in killing was seen between *ΔcopA/ΔgolT* and SL1344 by peroxynitrite. Peroxynitrite is not known to liberate copper and is not influenced by free or complexed copper which explains the lack of difference in survival between the two strains. Peroxynitrite is believed to readily react with CO_2 forming an intermediate molecule that breaks down to form carbon trioxide radical and nitrite (Romero *et al.* 1999). Nitrite can be converted into nitric oxide but evidently this does not occur under the experimental conditions performed in this study due to a *hmp* mutant exhibiting similar growth to wildtype. Hmp detoxifies $NO^{\cdot}$ and is not capable of modifying nitrite and does not have any difference in survival to SL1344. A *hmp* mutant tolerance to peroxynitrite has previously been reported to be similar to wildtype S. Typhimurium (Stevanin *et al.* 2002).

3.5.8 Conclusions

Copper internalised within S. Typhimurium is unable to catalyse Fenton chemistry, with copper homeostasis mutants exhibiting no difference in hydrogen peroxide tolerance to SL1344. A *copA/golT* double mutant has decreased growth in the presence of ASN, GSNO and NOC5/7 in comparison to SL1344, with a *copA* or *golT* single mutant exhibiting no difference to SL1344. Furthermore, expression of *copA* and *golT* is induced by GSNO and

NOC5/7 indicating reactive nitrogen species can potentially liberate copper from bound ligands within S. Typhimurium. Hence, it can be suggested that reactive nitrogen species and copper tolerance systems overlap as seen in previous work with *M. tuberculosis* and *N. gonorrhoeae*.

Chapter 4

Copper provides an antimicrobial role during *Salmonella* infection of macrophages

Salmonella targets macrophages and macrophage like cells to survive within when systemically infecting a host. To survive within macrophages *Salmonella* must avoid and detoxify several antimicrobial threats. The use of copper as an antimicrobial agent by the immune system is a current area of research. White *et al.* (2009) identified an increase in copper uptake and expression of copper transporters CTR1 and ATP-7a in response to infection of macrophages by *E. coli*. Osman *et al.* (2010) identified the presence of copper within the *Salmonella* containing vacuole by monitoring copper-responsive expression during infection and decreased growth of a *ΔcopA/ΔgolT* copper transporter mutant during infection. Achard *et al.* (2012) identified that infection of macrophages by S. Typhimurium also gave an increase in expression of copper transporters CTR1 as well as other proteins involved in copper handling; and reported copper hot-spots accumulating in intracellular vesicles around the *Salmonella* containing vacuole.

It remains unknown what the mechanism of copper supply within the *Salmonella* containing vacuole is. As previously stated reactive nitrogen species could liberate copper from bound ligands within *Salmonella*. Macrophages could also directly transport copper into the phagosome, as suggested for ATP-7a, or by the actions of a copper transporter or the fusion of copper containing vesicles to the *Salmonella* containing vacuole.

4.1 *ΔcopA/ΔgolT* has reduced growth within macrophages compared to SL1344

Initially, to confirm previous findings reported by Osman *et al.* (2010) competitive infections were performed by mixing SL1344 and a *ΔcopA/ΔgolT*.:cat overnight cultures together at a 1:1 ratio and used to infect Raw 264.7 macrophages at a 10:1 MOI (bacteria : macrophages). At various time points post infection, infected macrophages were washed with PBS to remove external bacteria and lysed with 0.9% Triton-X to release intracellular bacteria. Intracellular bacteria were quantified by serial dilution and plating out onto LB agar plates with and without chloramphenicol to quantify the number of SL1344 and *mutant* bacteria that contain a chloramphenicol resistance cassette. No difference in survival

between SL1344 and *ΔcopA/ΔgolT* was present at 2, 4 and 8 hours post infection. However, a difference in replication between the strains was evident at 24 hours post infection. There were significantly reduced numbers of *ΔcopA/ΔgolT* ($0.05 > p$) in comparison to SL1344 at 24 hours, with a viable count of 1.73×10^6 compared to SL1344 which had a viable count of 4.91×10^6, a 2.83 fold difference (figure 4.1). This confirms previous work that *Salmonella* is exposed to copper within the *SCV* and the ability to export copper via CopA or GolT provides a selective advantage to S. Typhimurium in this environment (Osman *et al.* 2010).

4.2 Activation of Raw 264.7 macrophages using IFN-γ to increase their antimicrobial potency

To further investigate the ability of S. Typhimurium to survive within macrophages, macrophages were activated within the pro-inflammatory cytokine IFN-γ. IFN-γ is routinely utilised to activate macrophages to increase their antimicrobial arsenal against intracellular pathogens and is an essential cytokine in amplifying a T helper cell 1 immune response against intracellular pathogens (Gilberthorpe *et al.* 2007, Herbst e*t al.* 2011). A titration was performed with increasing concentrations of IFN-γ added to macrophages 12-18 hours prior to incubation with SL1344 at concentrations of 0 U, 500 U and 1000 U ml^{-1} (figure 4.2). In resting macrophages an increase in SL1344 was detected between 8 and 24 hours (figure 4.1). A drop in SL1344 viability was seen using 500 U and 1000 U IFN-γ ml^{-1} activation between 8 and 24 hours. Treatment with 1000 U IFN-γ ml^{-1} gave a greater decrease in survival of SL1344 at 12 hours post infection than macrophages treated with 500 U IFN-γ, a 4 and 2 fold drop in bacterial numbers were obtained, respectively. From this point forward, pre-treatment with 1000 U ml^{-1} was used to activate macrophages prior to infection challenge with S. Typhimurium. To confirm that IFN-γ activated macrophages, an ELISA assay was performed to quantify the levels of IL-1β produced (figure 4.2). Macrophages were pre-treated with and without 1000 U IFN-γ ml^{-1} 12-18 hours prior to incubation with SL1344. At various time points, post infection, macrophages were washed and lysed by the addition of 0.9% Triton-X. Macrophage lysate and intracellular bacteria were collected and pelleted by centrifugation and supernatant was used in an IL-1β ELISA kit to detect IL-1β levels within macrophages. Macrophages activated with 1000 U IFN-γ ml^{-1} had reduced levels of IL-1β from 8 hours post infection compared to resting macrophages. The largest difference was observed at 24 hours post infection, activated macrophages had 414.39 pg ml^{-1} IL-1β compared to 582.13 pg ml^{-1} IL-1β present within resting macrophages. IL-1β levels are known to drop in mouse macrophages upon activation (Chujor *et al.* 1996). The lower

levels of IL-1β at 8 and 24 hours and reduced survival of SL1344 at 12 and 24 hours post infection indicates that macrophages are activated by 1000 U IFN-γ ml^{-1}.

4.2.1 *ΔcopA/ΔgolT* has reduced growth within IFN-γ activated macrophages compared to SL1344

To test the affect of activating Raw 264.7 macrophages with IFN-γ, competitive infections were performed with SL1344 and *ΔcopA/ΔgolT.:cat* in IFN-γ activated macrophages (figure 4.3). Unlike resting macrophages a drop in survival is seen for both strains at time points beyond 8 hours post infection, whereas in resting macrophages both strains are able to replicate 8 hours post infection (figure 4.1). Furthermore, *ΔcopA/ΔgolT.:cat* had reduced survival at 12 and 24 hours post infection compared to SL1344. To further investigate changes in survival of SL1344 and *ΔcopA/ΔgolT.:cat* competitive infections were repeated with time points included between 12 and 24 hours post infection (figure 4.3B).

The largest difference in survival between SL1344 and *ΔcopA/ΔgolT.:cat* was seen at 24 hours post infection with a 2.9 fold difference. In resting macrophages a 2.8 fold difference was present between SL1344 and *ΔcopA/ΔgolT.:cat* due to the ability of SL1344 to replicate with greater ability where as the difference exhibited during infections of activated macrophages is survival based. This identifies the ability to export copper is important for bacterial survival in macrophages with and without IFN-γ stimulation, indicating that elevated copper is a feature of both conditions. The addition of IFN-γ increases the antimicrobial arsenal of macrophages as seen by inhibition of growth and killing of S. Typhimurium.

To confirm that loss of both *copA* and *golT* is associated with reduced survival in IFN-γ activated macrophage infections, competitive infections were performed with SL1344 against the single mutant's *ΔcopA.:cat* and *ΔgolT.:cat* separately (figure 4.5). No difference in survival was seen between SL1344 and either of *ΔcopA.:cat* or *ΔgolT.:cat*. These data support the hypothesis that copper is the cause of reduced bacterial growth within macrophages from 12 hours post infection onwards. *ΔcopA.:cat* or *ΔgolT.:cat* do not exhibit reduced copper tolerance due to the functional redundancy between CopA and GolT in providing copper resistance, This indicated that the reduced viability of *ΔcopA/ΔgolT.:cat* in macrophages is associated with reduced copper resistance, and subsequent data has indentified a role for CopA and GolT in supplying copper to $SodC_{II}$ (Osman *et al.* 2013).

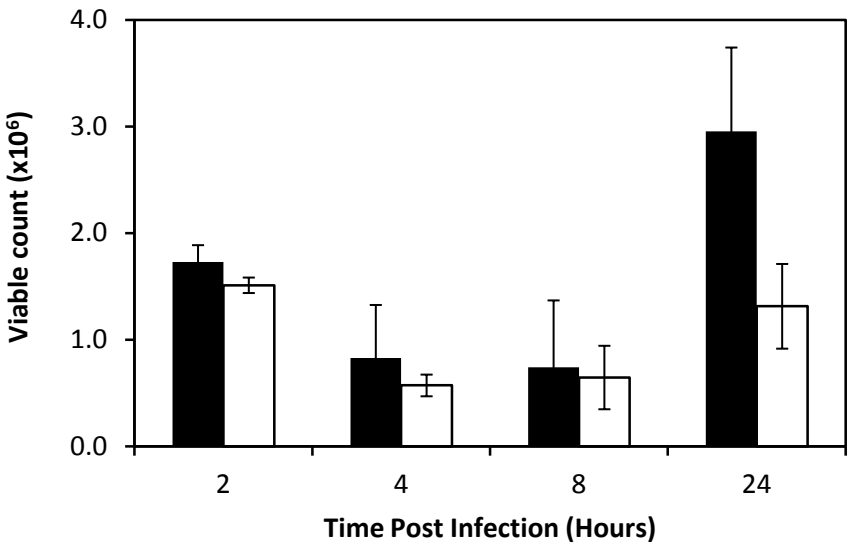


Figure 4.1 *ΔcopA/ΔgolT* has reduced growth within resting Raw 264.7 macrophages

Competitive infections were performed with SL1344 (black) and *ΔcopA/ΔgolT.:cat* (white) stationary phase bacterial overnight cultures (mixed 1:1) within Raw 264.7 macrophages at an MOI of 10:1 (bacteria : macrophage). At indicated time points macrophages were lysed with 0.9% Triton-X and intracellular bacteria were serially diluted in PBS and quantified by plating onto LB agar plates with and without 10 µg ml^{-1} chloramphenicol to distinguish between SL1344 and *ΔcopA/ΔgolT.:cat* by the method of Miles and Misra, and grown at 37°C statically overnight. Data points represent the mean of three independent repeats performed in triplicate, error bars represent standard error.

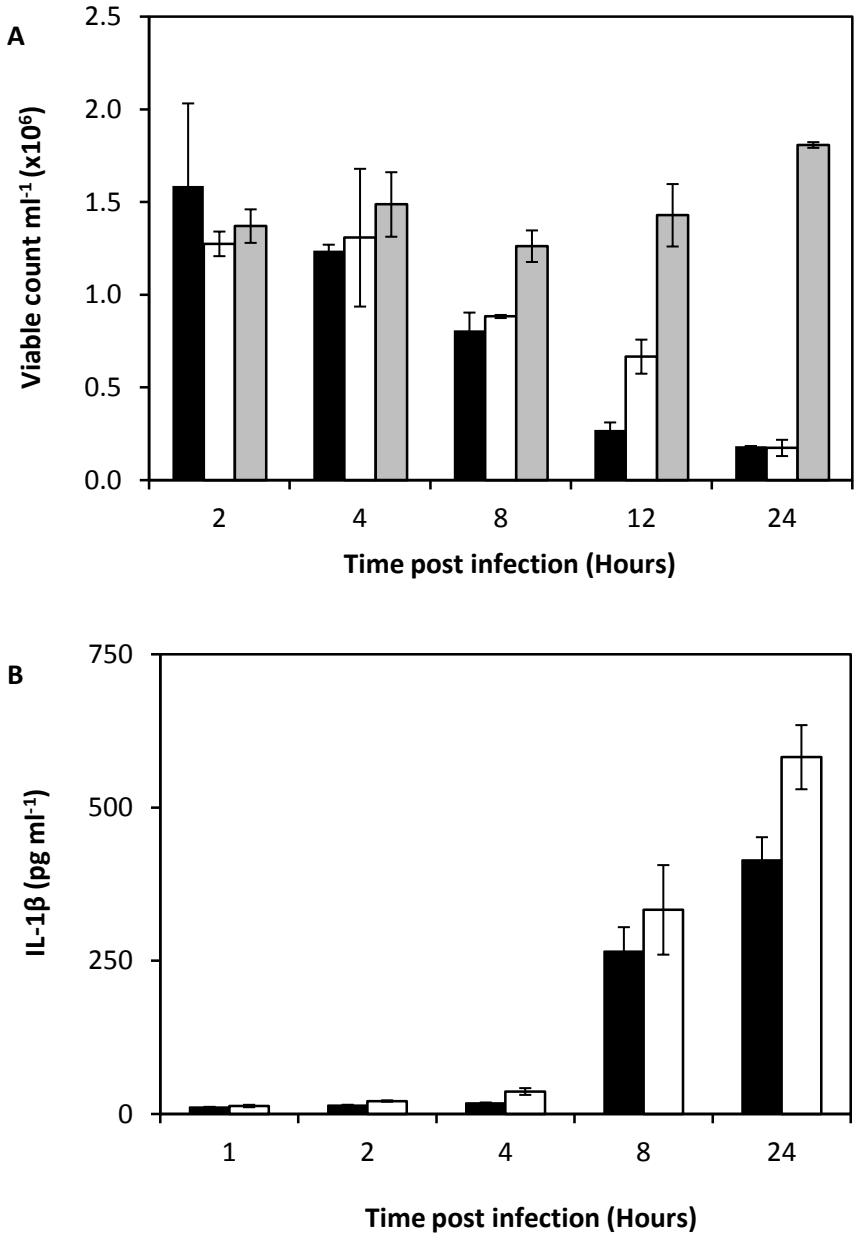


Figure 4.2 IFN-γ pre-treatment of macrophages prevents replication of S. Typhimurium

(A) Infections of Raw 264.7 macrophages using stationary phase SL1344 overnight culture using an MOI of 10:1 (bacteria : macrophage). Macrophages were pre-treated with: 0 U ml^{-1} (grey), 500 U ml^{-1} (white) and 1000 U ml^{-1} (black) IFN-γ. At indicated time points macrophages were lysed with 0.9% Triton-X and intracellular bacteria were serially diluted in PBS and quantified by plating onto LB agar plates with and without 10 µg ml^{-1} chloramphenicol to distinguish between SL1344 and *ΔcopA/ΔgolT.:cat* by the method of Miles and Misra, and grown at 37°C statically overnight. (B) 1000 U IFN-γ ml^{-1} activated macrophages (black bars) and resting (white bars) were lysed with 0.9% Triton-X, and IL-1β quantified by an Elisa detection kit. Data points represent the mean of one repeat performed in triplicate, error bars represent standard error.

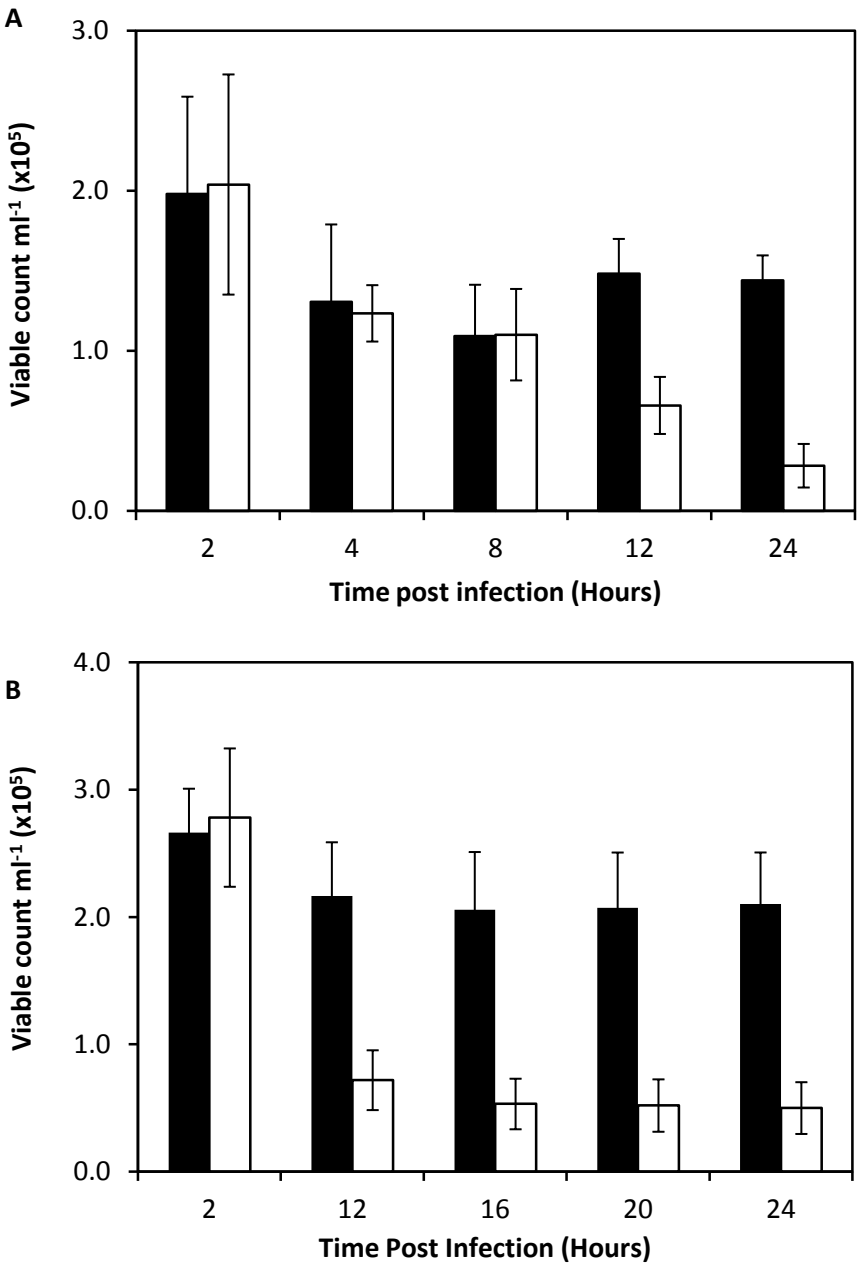


Figure 4.3 A *copA/golT* double mutant has reduced survival within IFN-γ activated Raw 264.7 macrophages between 12 and 24 hours post infection

Competitive infections were performed with SL1344 (black) and *ΔcopA/ΔgolT.:cat* (white) stationary phase bacterial overnight cultures (mixed 1:1) of 1000 U ml^{-1} IFN-γ activated Raw 264.7 macrophages at an MOI of 10:1 (bacteria : macrophage). At indicated time points, (A) focus prior to 12 hours, (B) focus after 12 hours, macrophages were lysed with 0.9% Triton-X and intracellular bacteria was serially diluted in PBS and quantified by plating onto LB agar plates with and without 10 μg ml^{-1} chloramphenicol to distinguish between SL1344 and *ΔcopA/ΔgolT.:cat* by the method of Miles and Misra, and grown at 37°C statically overnight. Data points represent the mean of three independent repeats performed in triplicate, error bars represent standard error.

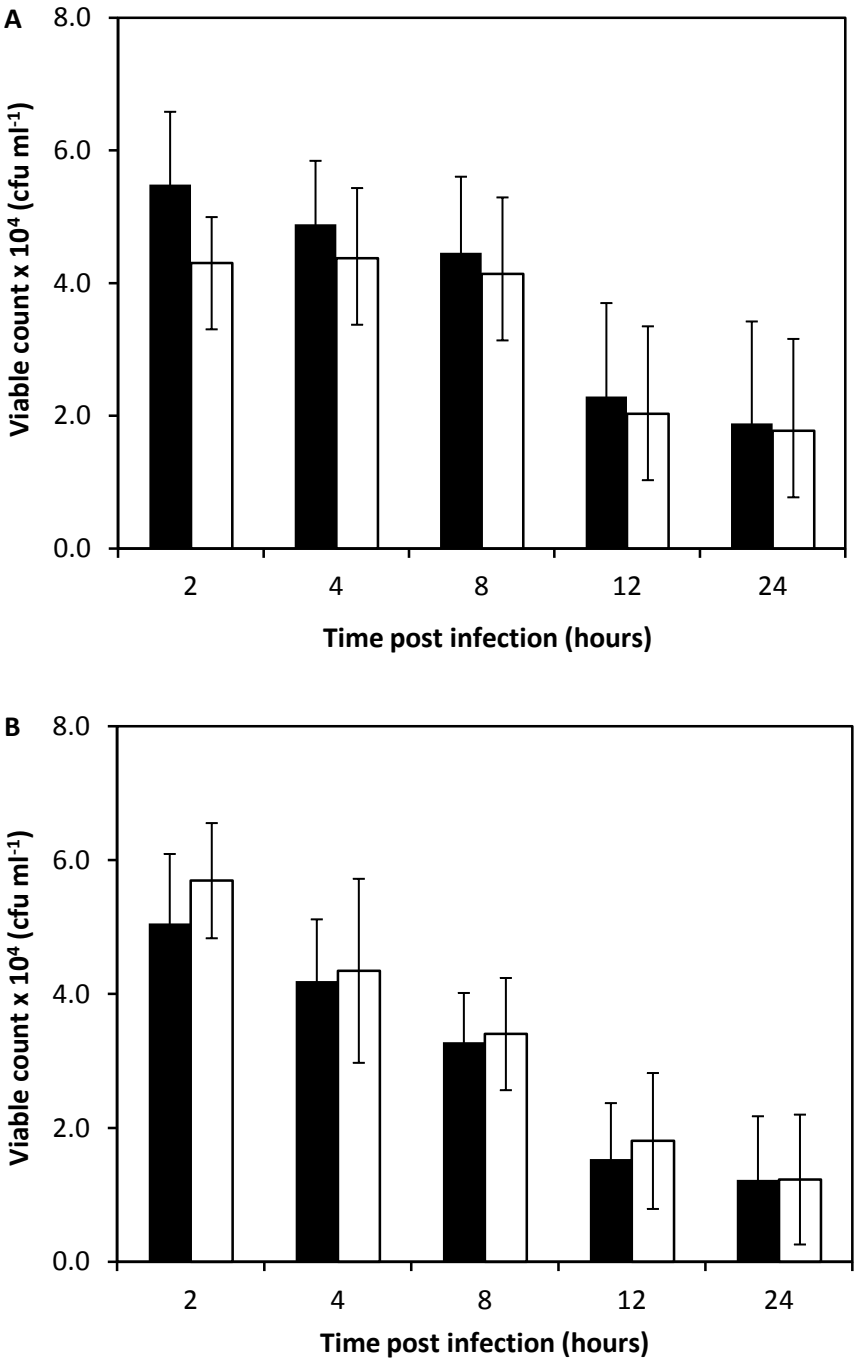


Figure 4.4 A *copA* or *golT* single mutant has no difference in survival within IFN-γ activated Raw 264.7 macrophages compared to SL1344

Competitive infections of SL1344 (black) and: (A) *ΔcopA.:cat*, (B) *ΔgolT.:cat* (white) stationary phase bacterial overnight cultures (mixed 1:1) within Raw 264.7 macrophages at an MOI of 10:1 (bacteria : macrophage). At indicated time points macrophages were lysed with 0.9% Triton-X and intracellular bacteria was serially diluted in PBS and quantified by plating onto LB agar plates with and without 10 μg ml^{-1} chloramphenicol to distinguish between SL1344 and *ΔcopA/ΔgolT.:cat* by the method of Miles and Misra, and grown at 37°C statically overnight. Data points represent the mean of three independent repeats performed in triplicate, error bars represent standard error.

4.3 IFN-γ activation of macrophages increases the production of nitrite

Reactive nitrogen species production can be estimated from the presence of nitrite within media containing infected macrophages by the addition to Griess reagent, which contains an azo-dye that gives a colorimetric change in the presence of nitrite. Previous work by Erikson *et al.* (2003) identified that reactive nitrogen species are produced by mouse macrophages approximately eight hours after infection.

The difference in replication and survival between SL1344 and *ΔcopA/ΔgolT.:cat* following infection of resting and IFN-γ pre-activated macrophages is seen beyond eight hours post infection, indicating reactive nitrogen species could contribute to the reduced survival of *ΔcopA/ΔgolT.:cat*. Reactive nitrogen species are capable of liberating copper from bound ligands in cells and copper mediated growth inhibition and/or killing of S. Typhimurium in macrophages could be reactive nitrogen species associated. A standard curve was performed mixing Griess reagent and known concentrations of sodium nitrite together at a 1:1 ratio and measuring the absorbance at 540 nm (figure 4.4). Supernatant was extracted from competitive infections of SL1344 and *ΔcopA/ΔgolT.:cat* between 0 and 24 hours post infection, and mixed with Griess reagent and absorbance at 540 nm measured. Using the standard curve, the concentration of nitrite was calculated (figure 4.4). Nitrite was detected from 12 hours post infection onwards but not before. IFN-γ activated macrophages produced significantly greater levels of nitrite than resting macrophages at 16, 20 and 24 hours post infection ($p<0.05$).

When competitive infection data for SL1344 and; *ΔcopA.:cat*, *ΔgolT.:cat* and *ΔcopA/ΔgolT.:cat* is compared to nitrite production a correlation in timings is seen (figure 4.6). Nitrite is first detected at 12 hours post infection, where *ΔcopA/ΔgolT.:cat* survival is reduced, and at 24 hours post infection, where the greatest difference in survival between SL1344 and *ΔcopA/ΔgolT.:cat* is detected the greatest concentration of nitrite is recorded, 74 µM.

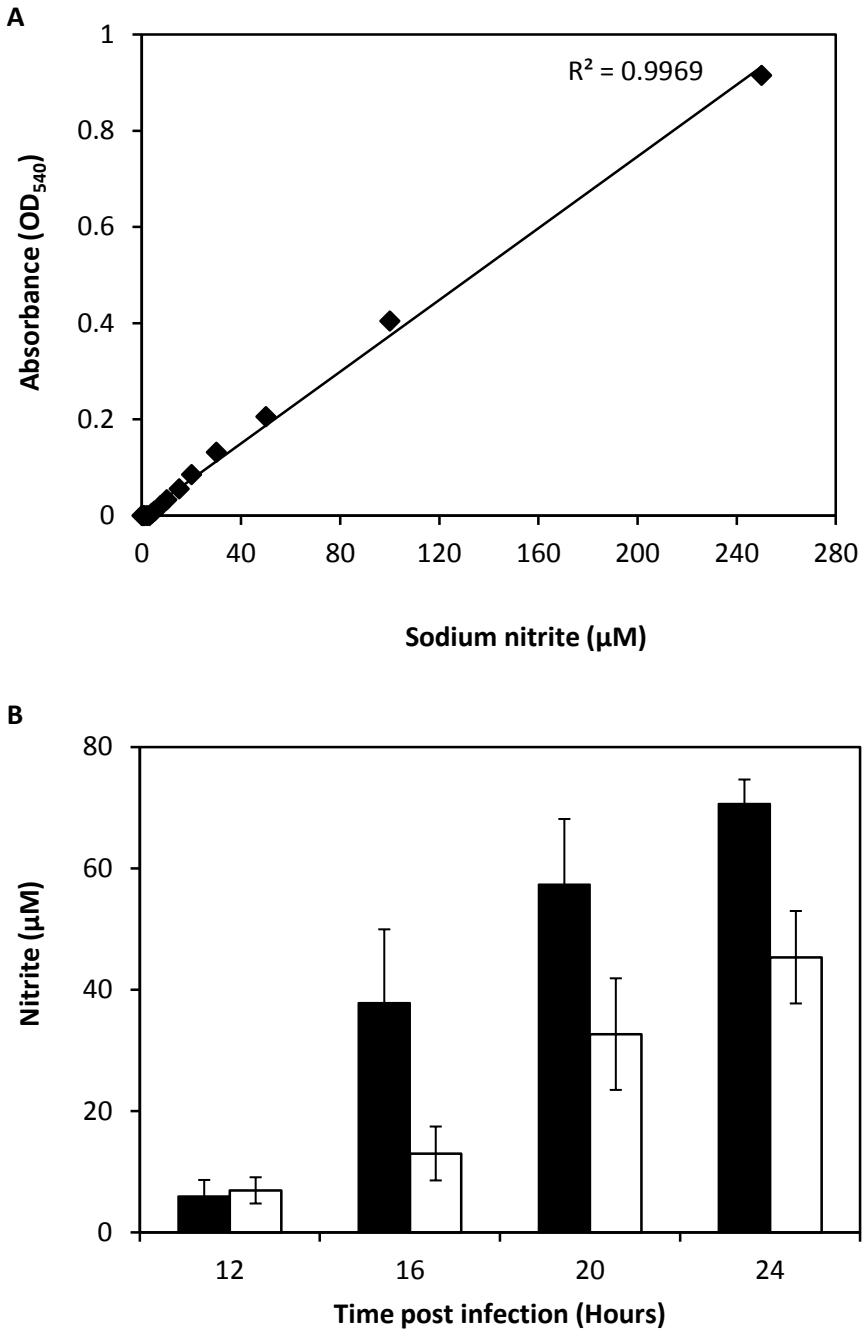


Figure 4.5 An increase in nitrate levels occurs between 12 and 24 hours post SL1344 infection of Raw 264.7 macrophages

Griess reagent was mixed 1:1 with culture to determine nitrite levels. (A) Sodium nitrite dissolved within DMEM to create a standard curve. (B) Media from infection wells using either pre-activated IFN-γ (black) or resting (white) macrophages prior to lysing with 0.9% Triton-X. Absorbance was measured at 540 nm. Data points represent the mean of three independent repeats performed in triplicate, error bars represent standard error.

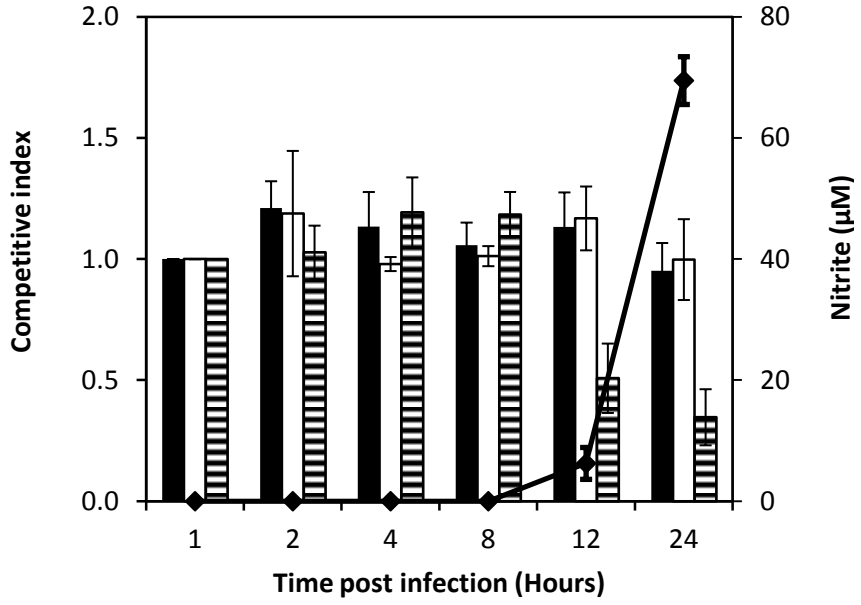


Figure 4.6 Reduced survival of *ΔcopA/ΔgolT* within IFN-γ pre-activated macrophages coincides with increased nitrite levels

Competitive index calculated from competitive infections with stationary phase bacterial overnight cultures of SL1344 versus: *ΔcopA.:cat* (black), *ΔgolT.:cat* (white) and *ΔcopA/ΔgolT.:cat* (stripes) (mixed 1:1) within IFN-γ activated Raw 264.7 macrophages at an MOI of 10:1 (bacteria : macrophage). At indicated time points macrophages were lysed with 0.9% Triton-X and intracellular bacteria was serially diluted in PBS and quantified by plating onto LB agar plates with and without 10 µg ml^{-1} chloramphenicol to distinguish between SL1344 and copper homeostasis mutants containing a chloramphenicol resistance cassette by the method of Miles and Misra, and grown at 37°C statically overnight. Griess reagent was mixed 1:1 with culture to determine nitrite levels at specified time points by measuring absorbance at 540nm (♦). Data points represent the mean of three independent repeats performed in triplicate, error bars represent standard error.

4.4 Inhibition of iNOS does not affect *ΔcopA/ΔgolT* survival within IFN-γ activated macrophages

Having identified nitrite production occurs at the same time that a reduction in survival of *ΔcopA/ΔgolT.:cat* is detected in macrophages, the contribution of iNOS activity to bacterial killing was examined. To test if reactive nitrogen species are responsible for the decreased viability of a *ΔcopA/ΔgolT.:cat* at 12 hours onwards post infection, iNOS was inhibited. Infections were therefore performed with macrophages treated with L-NMMA, a competitive inhibitor of iNOS, prior to infection. L-NMMA binds to iNOS distorting its active site preventing nitric oxide production. Initially, to confirm that nitric oxide production by iNOS was inhibited by L-NMMA, a titration of L-NMMA concentrations was added to macrophages and subsequently infected with SL1344, and media from infection wells was removed and mixed at a 1:1 ratio with Griess reagent and absorbance at 540 nm measured (figure 4.7). The increasing concentrations of L-NMMA reduced the level of nitrite detected at 24 hours post infection. At 5 mM L-NMMA concentration, no nitrite was detected and hence forth 5 mM L-NMMA was utilised to inhibit iNOS activity.

An additional control was performed to monitor toxicity of L-NMMA toward S. Typhimurium, SL1344 and *ΔcopA/ΔgolT.:cat* were grown for 24 hours in DMEM supplemented with and without 5 mM L-NMMA (figure 4.8). No difference was present in the viable counts for either SL1344 or *ΔcopA/ΔgolT.:cat* for growth in presence of L-NMMA, indicating L-NMMA is not harmful to S. Typhimurium. Previous work has identified that Raw 264.7 macrophages are not harmed by L-NMMA (Rosenberger and Finlay 2003, Griffon *et al.* 1999). Competitive infections of L-NMMA and IFN-γ treated macrophages were performed with SL1344 and *ΔcopA/ΔgolT.:cat* (figure 4.9). Notably, increased survival of SL1344 was detected in L-NMMA treated macrophages compared to untreated (compare figure 4.9 to figure 4.3), this is consistent with reduced killing potential of macrophages upon treated with L-NMMA, as previous reported by Chakravortty *et al.* (2002) and Umezawa *et al.* (1997).

The inhibition of iNOS did not reduce the level of killing of *ΔcopA/ΔgolT.:cat*, such that *ΔcopA/ΔgolT.:cat* still showed reduced survival compared to SL1344. However, a significant difference ($p<0.05$) between SL1344 and *ΔcopA/ΔgolT.:cat* was present at 12, 16, 20 and 24 hours post infection. In iNOS inhibited macrophages, SL1344 grew between 12 and 24 hours post infection where as *ΔcopA/ΔgolT.:cat* was unable to do so. The survival of *ΔcopA.:cat* and *ΔgolT.:cat* single mutants was also examined in competitive infections with SL1344 of L-NMMA treated macrophages (figure 4.10B). As with non L-NMMA treated macrophages, *ΔcopA.:cat* and *ΔgolT.:cat* showed similar survival to SL1344, whereas the difference between SL1344 and *ΔcopA/ΔgolT.:cat* is enhanced.

These data identified that reactive nitrogen species produced by iNOS are not required for reduced survival of *ΔcopA/ΔgolT.:cat* in macrophages. This finding suggests that reactive nitrogen species are not completely responsible for the release of copper in macrophages associated with *ΔcopA/ΔgolT.:cat* killing. It seems likely that the *Salmonella* containing vacuole receives copper directly either by the action of a copper transporter such as ATP-7a or the fusion of copper containing vesicles.

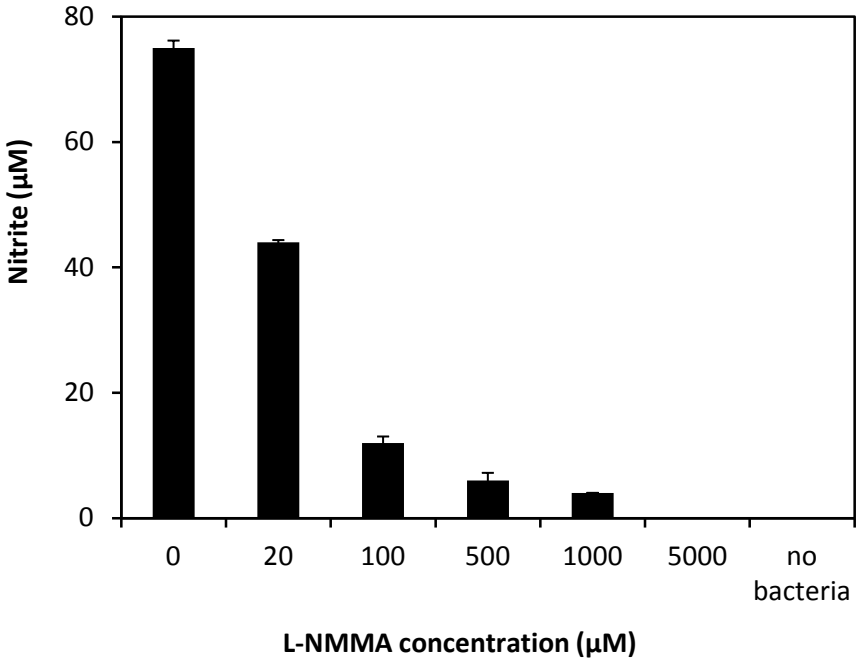


Figure 4.7 L-NMMA titration to inhibit the production of nitrite by Raw 264.7 macrophages

Competitive infections of L-NMMA treated Raw 264.7 macrophages by stationary phase SL1344 overnight culture. An MOI of 10:1 (bacteria : macrophage) was utilised. Griess reagent was mixed 1:1 with culture to determine nitrite levels after 24 hours. Data points represent the mean of three independent repeats performed in triplicate, error bars represent standard error.

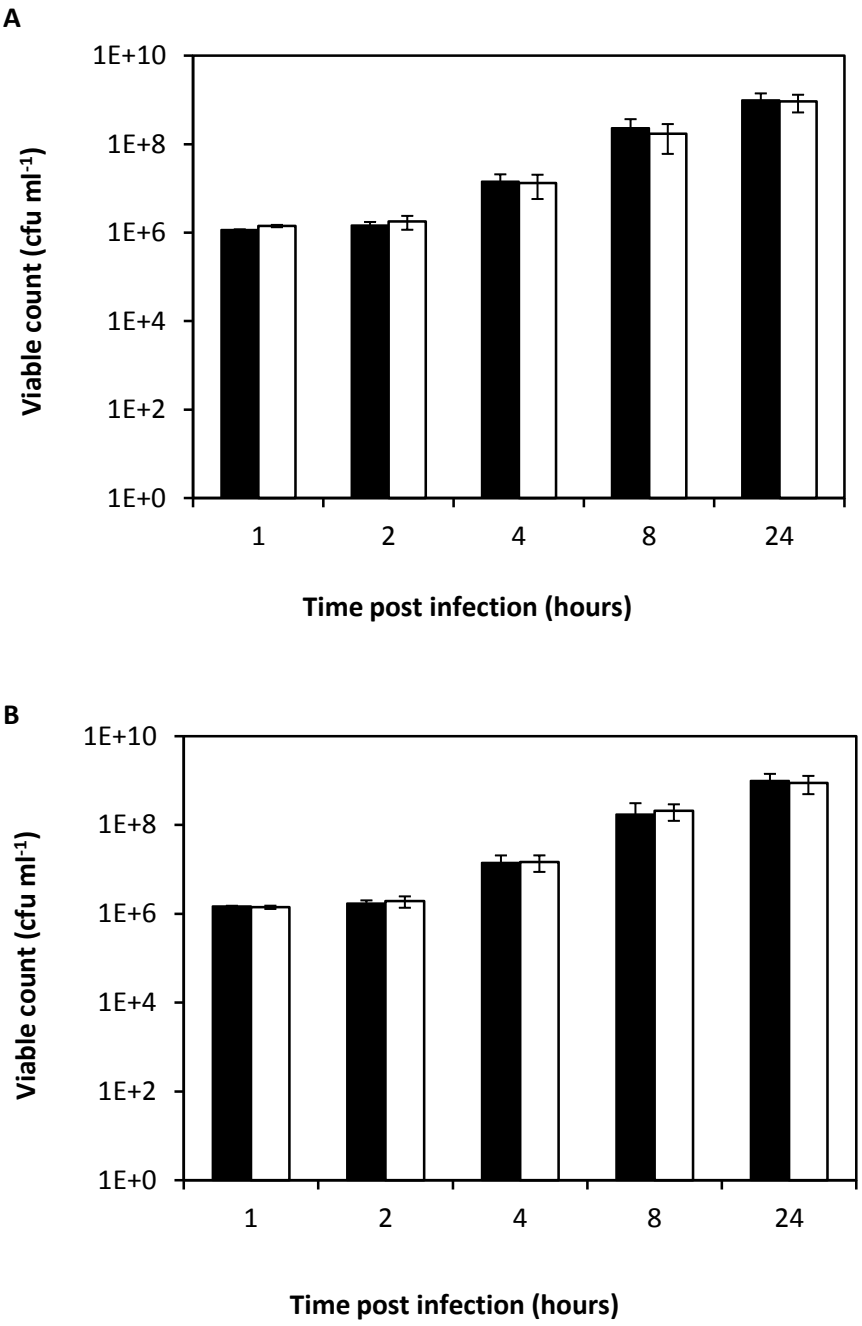


Figure 4.8 L-NMMA does not affect S. Typhimurium viability

SL1344 (black) and *ΔcopA/ΔgolT.:cat* (white) were grown in (A) DMEM and (B) DMEM supplemented with 5 mM L-NMMA at 37°C were serially diluted in PBS and quantified by plating onto LB agar plates with and without 10 μg ml^{-1} chloramphenicol to distinguish between SL1344 and *ΔcopA/ΔgolT.:cat* by the method of Miles and Misra, and grown at 37°C statically overnight. Data points represent the mean of three independent repeats performed in triplicate, error bars represent standard error.

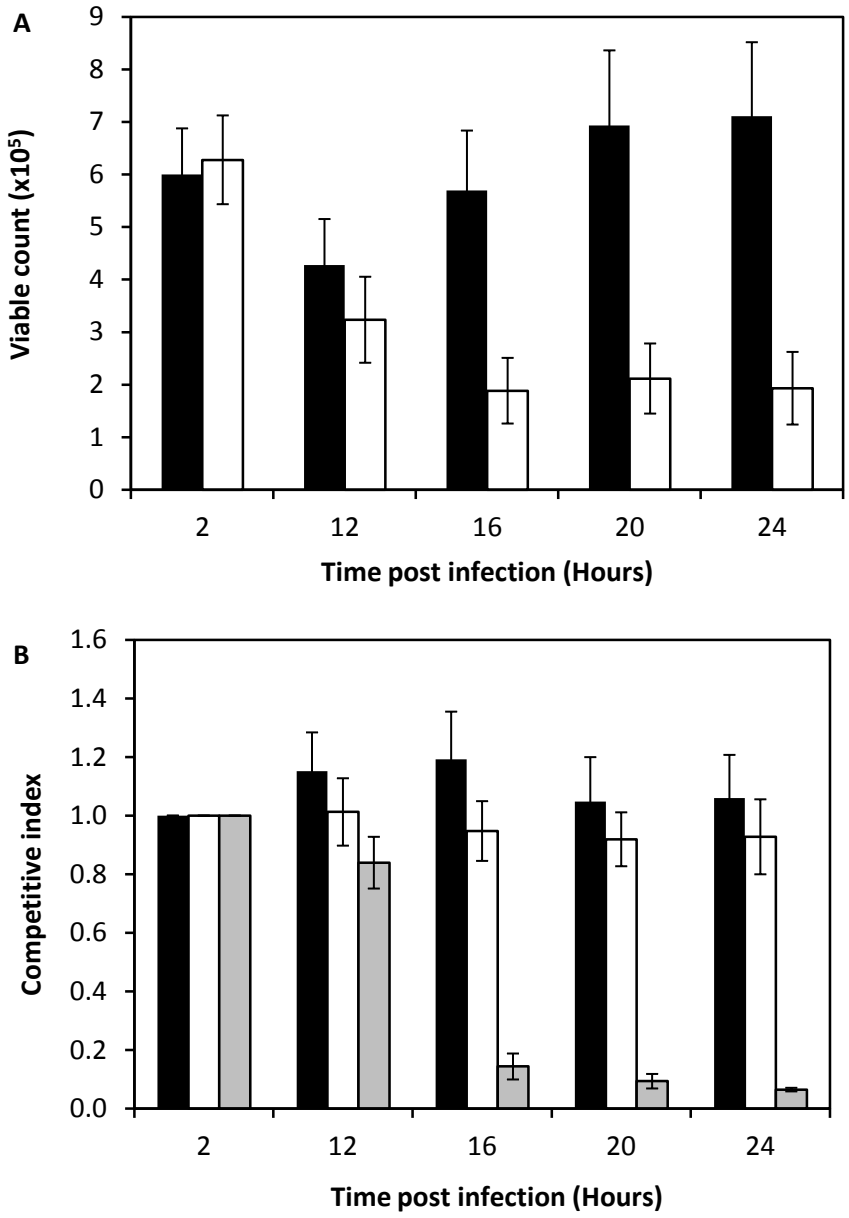


Figure 4.9 Inhibition of iNOS does not restore survival of the *copA/golT* double mutant during infection of Raw 264.7 macrophages

Competitive infections of (A) SL1344 (black) and *ΔcopA*, *ΔgolT.:cat* (white) stationary phase bacterial overnight cultures (mixed 1:1). (B) Competitive index of competitive infections of SL1344 against *ΔcopA.:cat* (black), *ΔgolT.:cat* (white) and *ΔcopA*/*ΔgolT.:cat* (grey). Infections were performed within Raw 264.7 macrophages pre-treated with 1000U ml^{-1} IFN-γ and 5 mM L-NMMA at an MOI of 10:1 (bacteria : macrophage). At indicated time points macrophages were lysed with 0.9% Triton-X and intracellular bacteria was serially diluted in PBS and quantified by plating onto LB agar plates with and without 10 µg ml^{-1} chloramphenicol to distinguish between SL1344 and copper homeostasis mutants containing a chloramphenicol resistance cassette by the method of Miles and Misra, and grown at 37°C statically overnight. Data points represent the mean of three independent repeats performed in triplicate, error bars represent standard error.

4.5 The addition of BCS to DMEM restores *ΔcopA/ΔgolT* viability to a similar viability as SL1344

To investigate the direct requirement of copper for the reduced survival of *ΔcopA/ΔgolT.:cat* during macrophage infection, macrophages were grown in DMEM containing the copper chelator BCS. Controls to ensure BCS was not harmful to S. Typhimurium and macrophages were also performed (figure 4.11). SL1344 and *ΔcopA/ΔgolT.:cat* were grown in the presence of BCS up to a concentration of 500 µM for 24 hours and viable counts were performed to quantify living bacteria. No difference in viable counts was present between cultures grown in DMEM and cultures grown in DMEM supplemented with 500 µM BCS. Similarly, macrophages showed similar viability in DMEM and DMEM supplemented with 500 µM BCS. Competitive infections using SL1344 and *ΔcopA/ΔgolT.:cat* were performed using resting, IFN-γ activated and L-NMMA pre-treated macrophages in DMEM containing 500 µM BCS (figure 4.12). The addition of BCS to resting, IFN-γ activated and L-NMMA pre-treated macrophages restored the survival of *ΔcopA/ΔgolT.:cat* to a similar level to that of SL1344. In BCS treated resting macrophages both SL1344 and *ΔcopA/ΔgolT.:cat* grew to a similar viable count. Whereas, in the absence of BCS both SL1344 and *ΔcopA/ΔgolT.:cat* replicated but *ΔcopA/ΔgolT.:cat* replicated significantly less (figure 4.1). Previous infections with IFN-γ activated macrophages gave significantly greater killing of *ΔcopA/ΔgolT.:cat* than SL1344 (figure 4.3). Whereas, the addition of BCS resulted in both SL1334 and *ΔcopA/ΔgolT* having reduced killing in IFN-γ activated macrophages; and again restored survival of *ΔcopA/ΔgolT.:cat* to a similar level to SL1344. Whilst L-NMMA treatment of macrophages reduced the bactericidal activity against SL1344, this was not the case for *ΔcopA/ΔgolT.:cat* (figure 4.9). However treatment with BCS and L-NMMA resulted in substantial growth of both SL1344 and *ΔcopA/ΔgolT.:cat*. The addition of BCS therefore enables *ΔcopA/ΔgolT.:cat* to survive to a similar level as SL1344 and combined with the lack of reactive nitrogen species both strains of bacteria are able to replicate approximately 12 hours post infection. These data confirmed that macrophages need to uptake copper from the external environment to provide an antimicrobial copper response to S. Typhimurium. Taken together these data suggest copper associated killing in macrophages is not associated with reactive nitrogen species. S. Typhimurium copper export ability does not confer a selective advantage in BCS treated macrophages, consistent with macrophage bactericidal activity being directly associated with extracellular copper availability.

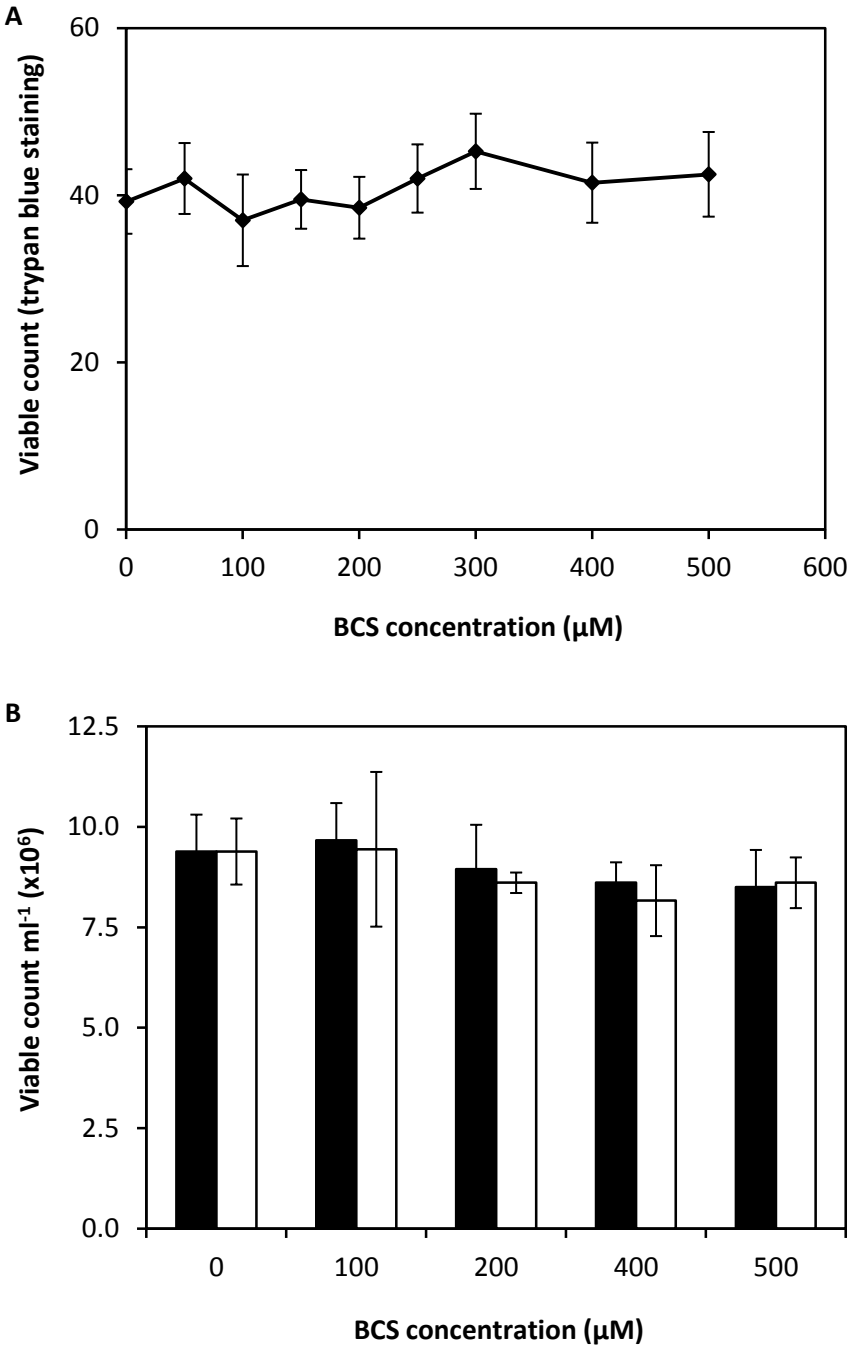
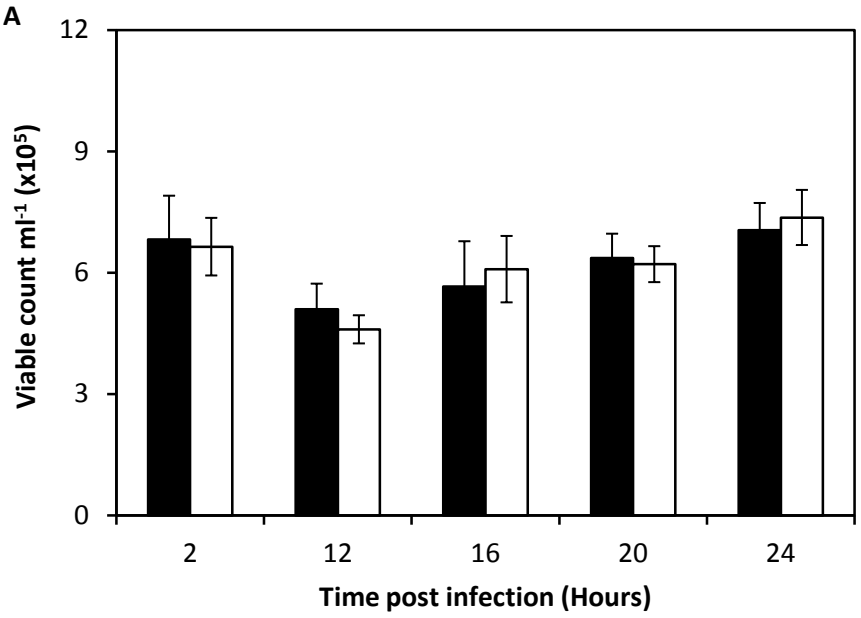
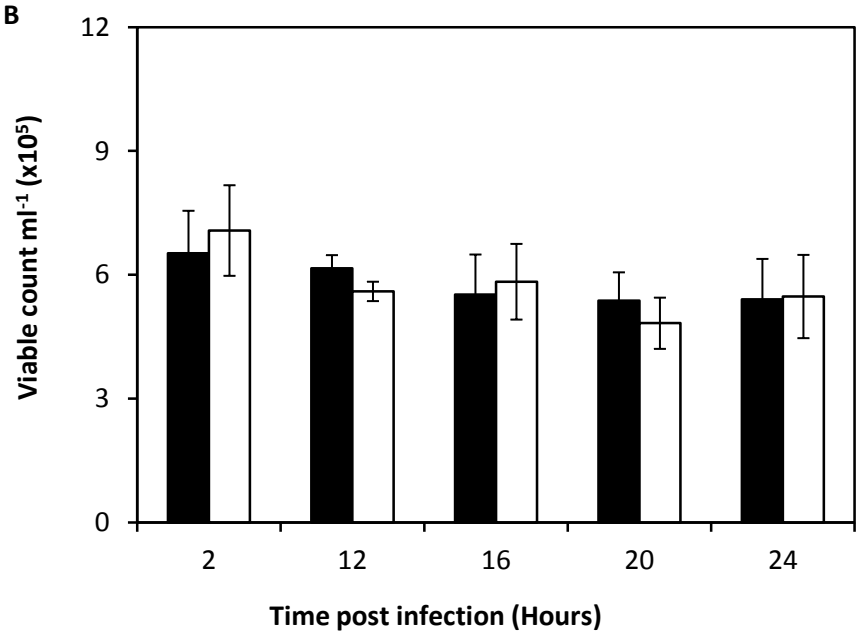


Figure 4.10 BCS does not affect Raw 264.7 macrophage or S. Typhimurium viability

(A) Raw 264.7 macrophages (B) overnight cultures of SL1344 (black) and *ΔcopA/ΔgolT.:cat* (white) were incubated in the presence of BCS at 37°C for 24 hours. Macrophages were re-suspended and mixed 1:1 with trypan blue and viability quantified under inspection by microscopy. Bacteria were serially diluted in PBS plated out onto LB agar with and without 10 µg ml^{-1} chloramphenicol to distinguish between SL1344 and *ΔcopA/ΔgolT.:cat* by the method of Miles and Misra, and grown at 37°C statically overnight. Data points represent the mean of three independent repeats performed in triplicate, error bars represent standard error.


A
12
9
6
3
0
Viable count ml-1 (x105)
2
12
16
20
24
Time post infection (Hours)


B
12
9
6
3
0
Viable count ml-1 (x105)
2
12
16
20
24
Time post infection (Hours)

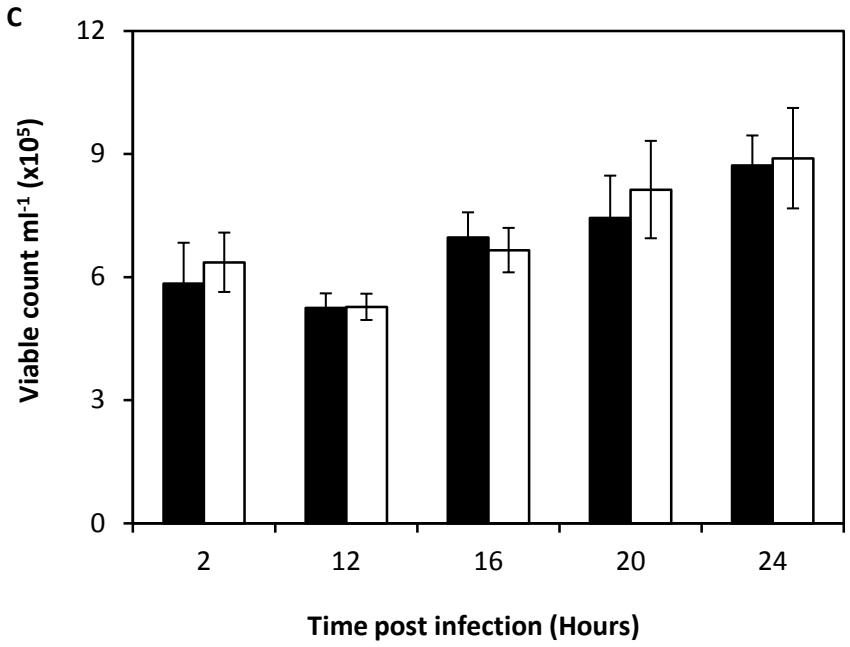


Figure 4.11 The addition of BCS to macrophage infections with S. Typhimurium restores survival of *ΔcopA/ΔgolT* to a similar level to SL-1344.

Competitive infections of SL1344 (black) and: *ΔcopA*/*ΔgolT.:cat* (white) stationary phase bacterial overnight cultures (mixed 1:1) within (A) resting, (B) IFN-γ activated and (C) L-NMMA treated, Raw 264.7 macrophages grown in the presence of 500 µM BCS and added at an MOI of 10:1 (bacteria : macrophage). At indicated time points macrophages were lysed with 0.9% Triton-X and intracellular bacteria was serially diluted in PBS and quantified by plating onto LB agar plates with and without 10 µg ml^{-1} chloramphenicol to distinguish between SL1344 and *ΔcopA/ΔgolT.:cat* by the method of Miles and Misra,and grown at 37°C statically overnight. Data points represent the mean of three independent repeats performed in triplicate, error bars represent standard error.

4.6 IFN-γ and LPS increase copper uptake within Raw 264.7 macrophages

Having identified that macrophages require an external source of copper for bactericidal activity, the copper quotas of pre-treated macrophages were investigated. Macrophages were untreated and treated with; IFN-γ, LPS, L-NMMA and BCS (figure 4.13). Resting macrophages had a copper quota of 12.48 (±0.59) $x10^3$ atoms per cell. The pre-treatment of macrophages with IFN-γ and LPS to macrophages increased the macrophage copper quota slightly, whilst pre-treatment with both LPS and IFN-γ raised macrophage copper quota further to 16.84 (±1.03) $x10^3$ atoms per cell. No difference was seen for copper quotas between IFN-γ treated macrophages (14.09 (±0.63) $x10^3$ atoms per cell) and macrophages treated with IFN-γ and L-NMMA (13.90 (±1.02) $x10^3$ atoms per cell) confirming that L-NMMA does not influence copper levels within macrophages. Macrophages grown in the presence of BCS had a significantly lower copper quota of 3.04 (±0.25) $x10^3$ atoms per cell.

At 24 hours post infection of a SL1344 and *ΔcopA/ΔgolT.:cat* competitive infection a competitive index of 0.35 (±0.05) was obtained in IFN-γ activated macrophages where as in resting macrophages a competitive index of 0.39 (±0.04) was given. The small increase in total copper quota for activated macrophages in comparison to resting macrophages presumably corresponds to the small detectable difference in the competitive index between resting and IFN-γ activated macrophages.

IFN-γ activation of macrophages increases the antimicrobial abilities of macrophages but does not influence a copper mediated antimicrobial response as strongly as other antimicrobial defences such as reactive oxygen species and reactive nitrogen species. If the copper mediated response was augmented by IFN-γ pre-activation of macrophages a higher copper quota within macrophages and a lower competitive index for *ΔcopA/ΔgolT.:cat* would be expected in IFN-γ treated macrophages compared to resting macrophages. Incubation of macrophages with BCS significantly lowered the copper quota of macrophages. The low level of intracellular copper within BCS pre-treated macrophages supports why reduced growth of *ΔcopA/ΔgolT.:cat* was not detected compared to SL1344 within BCS treated macrophages. Hence, supports a model in which copper export by CopA and GolT provides a selective advantage for S. Typhimurium in macrophages due to conferring copper resistance to protect against copper toxicity.

An additional control was performed to confirm the use of BCS within DMEM did not alter the levels of nitrite measured within DMEM. IFN-γ activated and IFN-γ + BCS treated macrophages had similar nitrite levels 62.00 µM (±3.50) and 56.33 µM (± 3.09) 24 hour post infection (figure 4.13).

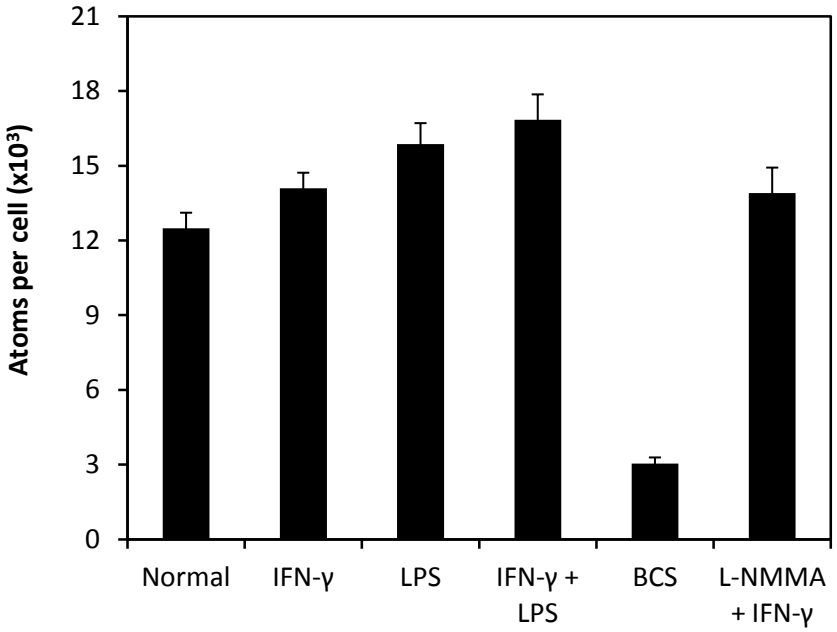


Figure 4.12 IFN-γ and LPS activation of Raw 264.7 macrophages increase the internal levels of copper

Raw 264.7 macrophages were supplemented with 1000 U IFN-γ ml^{-1}, 1 ng ml^{-1} LPS, 5 mM L-NMMA and 500 µM BCS and incubated at 37°C for 48 hours. Macrophages were washed with EDTA and lysed with nitric acid and intracellular metal levels quantified by ICP–MS analysis relative to number of atoms per cell. Data points represent the mean of three independent repeats performed in triplicate, error bars represent standard error.

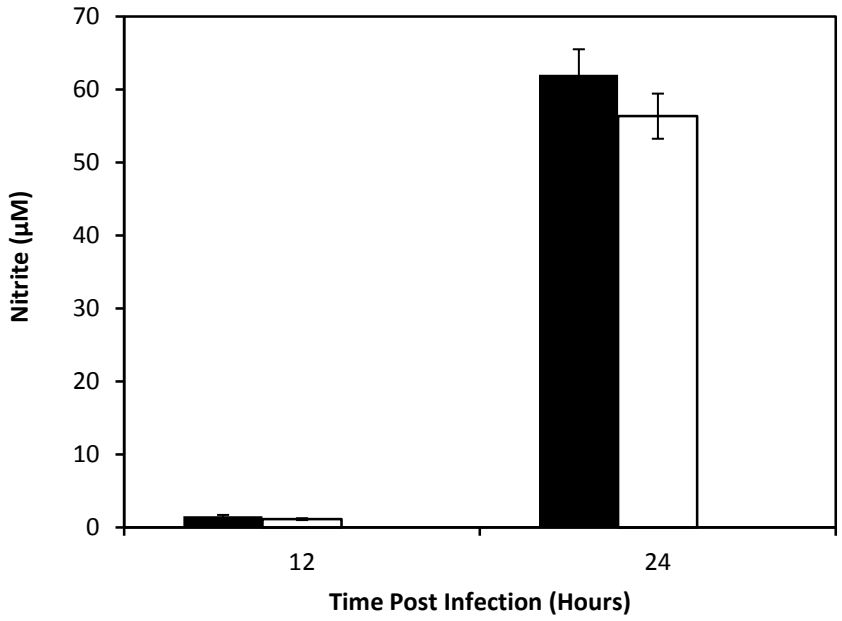


Figure 4.13 No significant difference in nitrite levels after BCS treatment of DMEM after IFN-γ activation of Raw 264.7 macrophages

Competitive infections of 1000U ml-1 IFN-γ pre-activated (black), 500 µM BCS treated (white) and 5mM L-NMMA treated (grey) Raw 264.7 macrophages by stationary phase SL1344 overnight culture, An MOI of 10:1 (bacteria : macrophage) was utilised. Griess reagent was mixed 1:1 with culture to determine nitrite levels after12 and 24 hours. Data points represent the mean of three independent repeats performed in triplicate, error bars represent standard error.

4.7 Expression of *copA* and *golT* occurs within S. Typhimurium during infection of IFN-γ activated and L-NMMA supplemented macrophages but not BCS treated macrophages

To further test the requirement of CopA and GolT during macrophage infections qRT-PCR was performed on S. Typhimurium extracted from macrophages to quantify the levels of expression of *copA* and *golT*. Previous work by Osman *et al.* (2010) has identified that *copA* and *golT* are both responsive to copper and that *copA* and *golT* are induced during S. Typhimurium intracellular infection of macrophages. Housekeeping genes of *rpoD* and *16s rRNA* were chosen to compare the change in *copA* and *golT* expression against. Control experiments were performed to ensure that experimental technique was performed correctly and that data acquired was accurate. SL1344 was grown in DMEM for 24 hours in parallel to infection experiments and *copA* and *golT* expression was monitored (figure 4.14). No significant fold change in gene expression was seen for *copA* or *golT* between 2 and 24 hours growth in DMEM. This indicated that growth in DMEM alone did not significantly alter the expression of *copA* or *golT*. A serial dilution was performed with each real-time PCR repeat to ensure accuracy of pipetting with genomic DNA (figure 4.15). The increase of the critical threshold value occurring with a decrease in DNA concentration confirms accuracy of pipetting. It would be expected that a greater number of PCR cycles are required before a lower concentration of DNA reaches a specific level of absorbance than a higher starting DNA concentration. After each real-time PCR cycle finished a melting curve was performed to analyse that the double stranded DNA (dsDNA) products formed during the PCR (figure 4.16). The melting curve would identify if any non-specific products were formed during the PCR due to the non-specific nature of SYBR-green dye binding to dsDNA. The presence of a single peak indicates that there is only one product produced during the PCR and the data is reliable.

Infections were performed with SL1344 within macrophages treated with IFN-γ, IFN-γ + L-NMMA and IFN-γ + BCS (figure 4.14). Expression of *copA* and *golT* were significantly raised ($p<0.05$) in both IFN-γ treated and in IFN-γ and L-NMMA treated macrophages in comparison to IFN-γ + BCS. Greater levels of expression were seen at 24 hours post infection than 12 hours post infection and that *copA* expression levels were higher than that of *golT*. Infection of IFN-γ activated macrophages at 24 hours post infection gave a *copA* gene fold change of 10.25 (±2.95) fold relative to *16s* expression and 13.40 (±3.09) fold relative to *rpoD*. Expression of *golT* increased 6.00 (±2.13) fold relative to *16s* expression and 7.09 (±2.29) fold relative to *rpoD*. Infection of IFN-γ and L-NMMA supplemented macrophages also exhibited increased levels of *copA* and *golT* expression with *copA* again

having a higher fold increase than *golT*. Expression of *copA* increased 9.53 (±1.14) relative to *16s* expression and 9.92 (±2.47) relative to *rpoD* expression. Expression of *golT* increased 7.46 (±3.21) relative to *16s* expression and 6.22 (±3.41) relative to *rpoD* expression. This indicates that S. Typhimurium is exposed to copper in macrophage phagosomes even when iNOS activity is inhibited. Infection of BCS treated macrophages gave a slight increase in *copA* and *golT* expression. Expression of *copA* increased 0.75 (±0.36) fold relative to *16s* expression and 0.51 (±0.36) fold relative to *rpoD* expression. Expression of *golT* increased 0.98 (±0.34) fold relative to*16s* expression and 0.44 (±0.02) fold relative to *rpoD* expression. The supplementation of BCS to DMEM significantly reduced the level of copper S. Typhimurium is exposed to during infections of macrophages and consequently only a slight increase in *copA* and *golT* expression is seen. This confirmed the requirement of copper in DMEM for a copper antimicrobial response to be mounted against intracellular S. Typhimurium and the requirement for S. Typhimurium copper export.

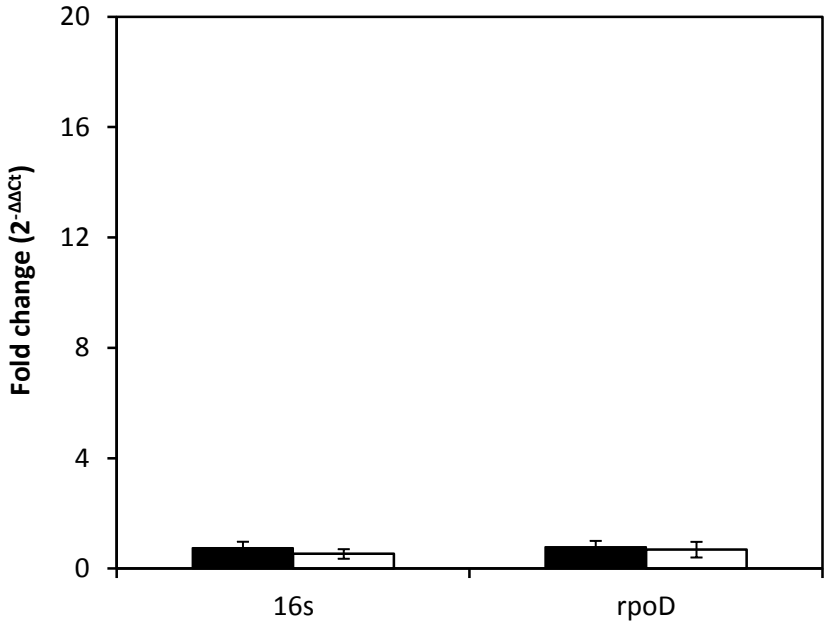


Figure 4.14 No significant change in gene expression of *copA* or *golT* in SL1344 grown in DMEM

An overnight culture of SL1344 was diluted 1/100 in DMEM and grown for 24 hrs at 37°C. Bacteria was pelleted and RNA protect was added to to re-suspend and perform RNA extraction. RT-PCR was quantified against *16s* and *rpoD* expression for *copA* (black) and *golT* (white). Data points represent the mean of three independent repeats performed in triplicate, error bars represent standard error.

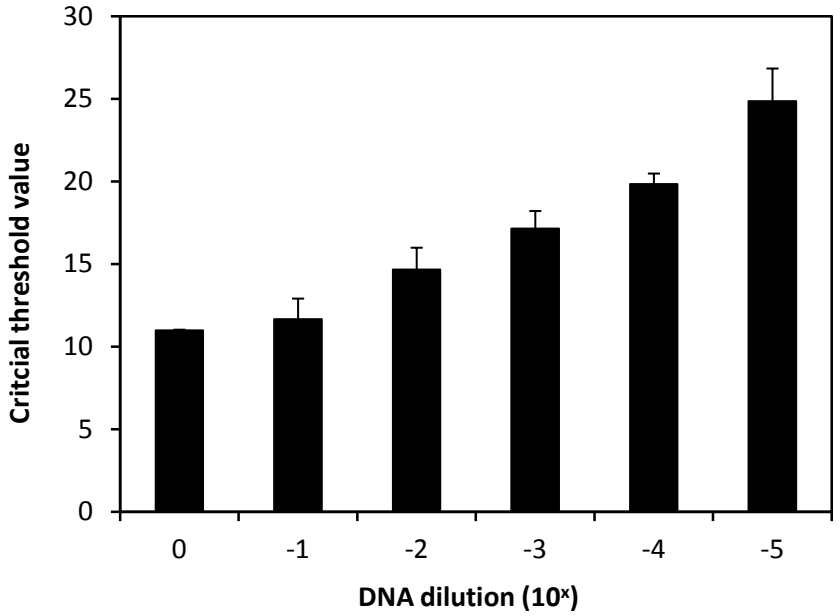


Figure 4.15 Standard curve using genomic DNA as a template confirmed accurate pipetting and increase of critical threshold values during real-time PCR

Genomic DNA was extracted from an overnight culture of SL1344 grown in DMEM and diluted within H_2O. RT-PCR was performed using genomic DNA as a template with 16s primers. Data points represent the mean of three independent repeats performed in triplicate, error bars represent standard error.

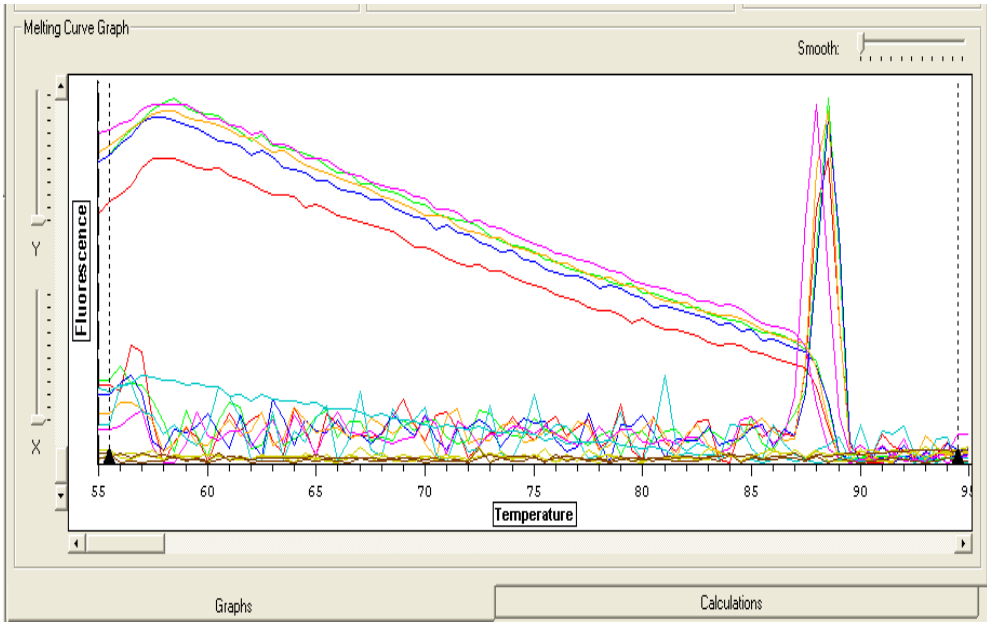
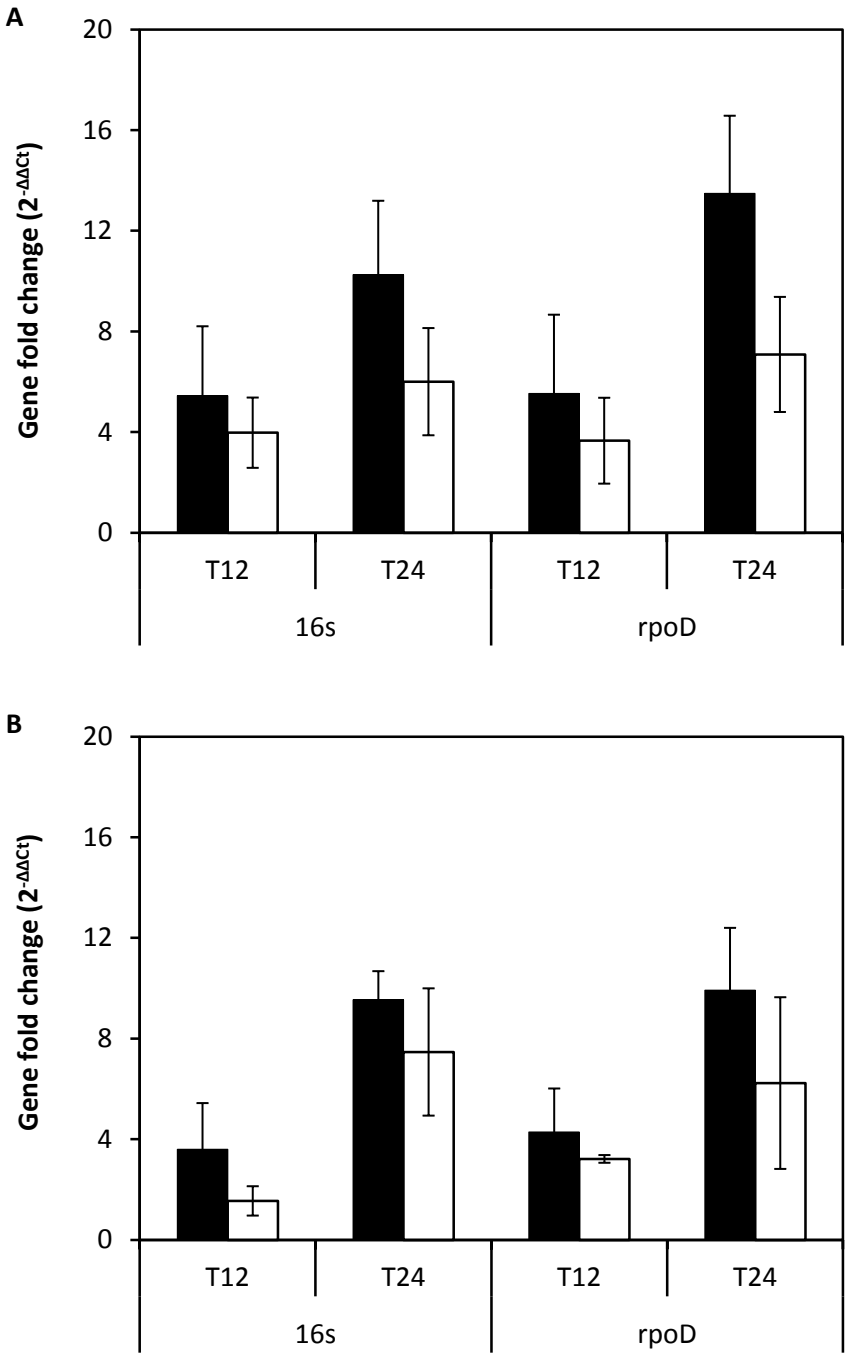


Figure 4.16 Melting curve confirms RT-PCR product was specific

Temperature was increased incrementally 0.5°C per second from 55°C until 95°C. SYBR Green dye was utilised to monitor PCR product formation during Real time PCR, SYBR green binds non-specifically to dsDNA. The melting curve provides a control to show that the PCR product formed during real time PCR is of a similar melting temperature consistent with a single product being analysed during real time PCR. The peaks measured during real time PCR have a melting temperature of between 87 - 90°C indicative of a single product being formed. If alternate peaks were measured at differing temperatures it would indicate that the PCR product measured during real time PCR is not an accurate measure of the gene of interest. Data is a representative of three independent repeats.


A
Gene fold change (2-ΔΔCt)
20
16
12
8
4
0
T12
T24
T12
T24
16s
rpoD
B
Gene fold change (2-ΔΔCt)
20
16
12
8
4
0
T12
T24
T12
T24
16s
rpoD

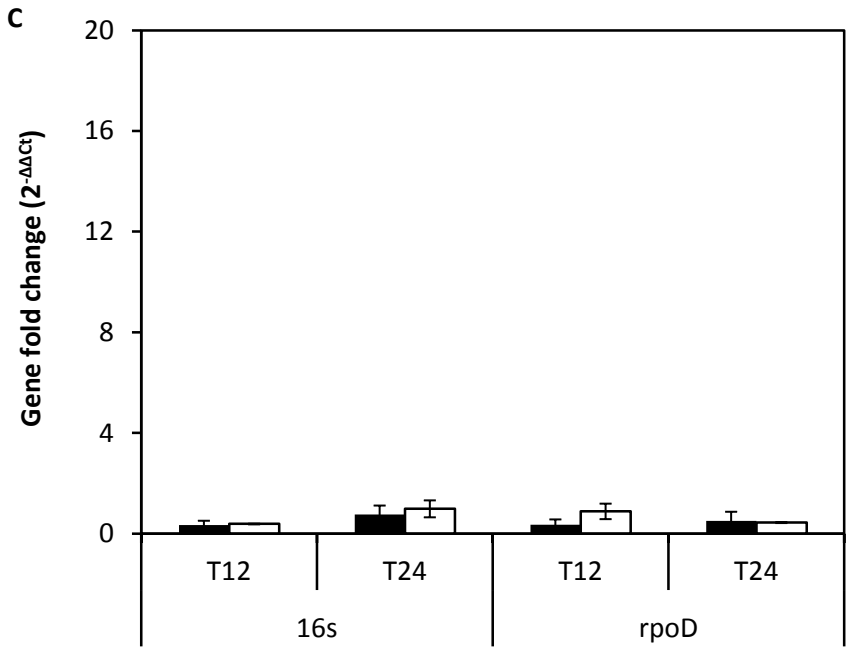


Figure 4.17 Expression of *copA* and *golT* is elevated during SL1344 infections of IFN-γ activated and L-NMMA treated macrophages but not during infection of BCS treated macrophages

Infections of Raw 264.7 macrophages by stationary phase SL1344 overnight culture using an MOI of 10:1 (bacteria : macrophage). Macrophages were treated with IFN-γ alone (A) IFN-γ and L-NMMA (B) and IFN-γ and BCS (C). RNA protect was added to infection wells prior to re-suspension and RNA extraction. RT-PCR was quantified against *16s* and *rpoD* expression for *copA* (black) and *golT* (white) at 12 and 24 hrs. Data points represent the mean of three independent repeats performed in triplicate, error bars represent standard error.

4.8 Light microscopy confirms reactive nitrogen species cause elongation of S. Typhimurium and identifies copper stress also initiates filamentation

Reactive nitrogen species have been reported to cause the filamentation of S. Typhimurium (Schapiro *et al.* 2003). The ability of reactive nitrogen species to cause filamentation of S. Typhimurium was tested with GSNO and NOC5/7. SL1344 and *ΔcopA*/*ΔgolT* were grown in the presence of GSNO and NOC5/7 overnight and the overnight culture was visualised under a light microscope (figure 4.17). Slight elongation was seen by SL1344 and *ΔcopA*/*ΔgolT* when treated with GSNO in comparison to SL1344 and *ΔcopA*/*ΔgolT* grown in LB media. Sporadic filamentous bacteria were seen in both SL1344 and *ΔcopA*/*ΔgolT* bacteria grown in the presence of NOC5/7. This confirmed previous work that reactive nitrogen species can cause filamentation of S. Typhimurium. No difference in overnight culture viability of SL1344 and *ΔcopA*/*ΔgolT* was present between LB media or LB media supplemented with GSNO or NOC5/7 (data not shown).

The morphology of S. Typhimurium in response to copper was also investigated. Overnight cultures of SL1344 were grown in the presence of copper and BCS as indicated (figure 4.18). SL1344 was grown in a high copper concentration (7.5 mM), which is slightly inhibitory to growth, and induces filamentation (figure 4.19). When BCS was added, in addition to copper overnight cultures filamentation was not seen and viability was restored to a level similar to an LB overnight culture (figure 4.19). This indicates that copper is capable of disrupting DNA replication and triggering a filamentous response within S. Typhimurium.

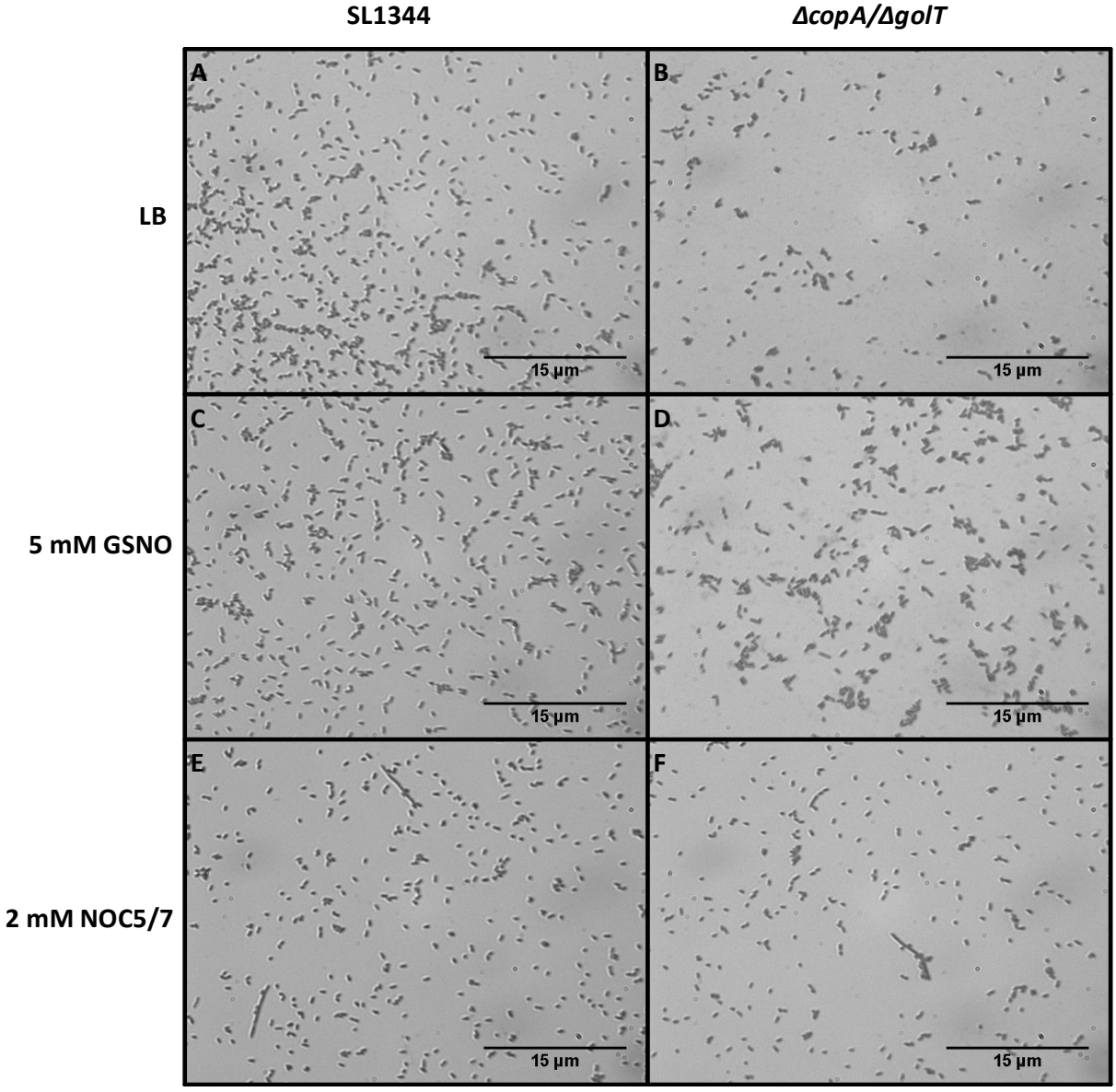


Figure 4.18 GSNO and NOC cause filamentation of S. Typhimurium

Overnight cultures of SL1344 and *ΔcopA/ΔgolT* were grown in the presence of (A,B) LB media and with the addition of (C,D) 5 mM GSNO and (E,F) 2 mM NOC5/7 at 37°C 200 rpm. Overnight cultures were stained with crystal violet and imaged at x100 oil immersion light microscopy. Data is a representative of three independent repeats performed in triplicate.

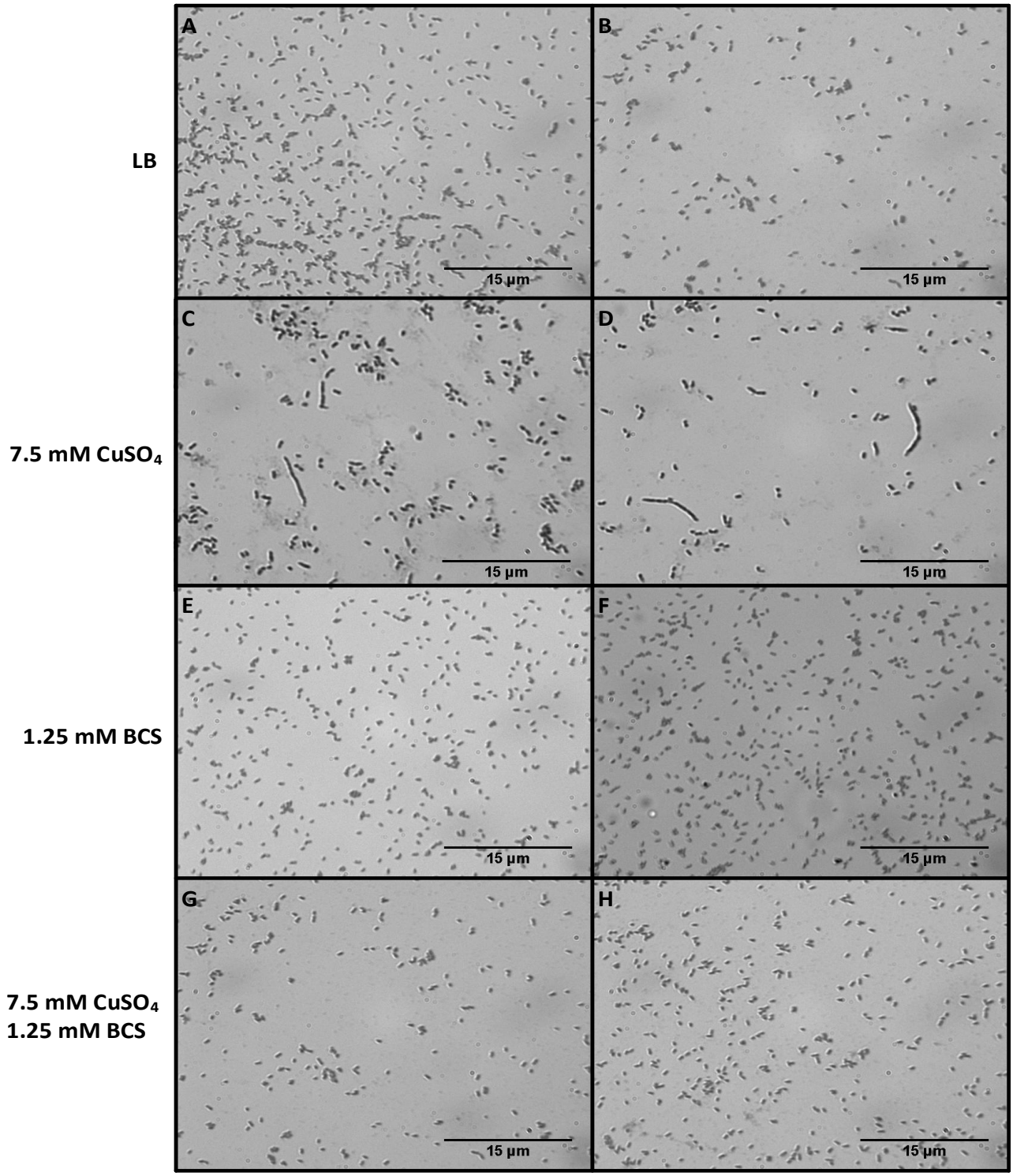


Figure 4.19 Copper can cause the filamentation of S. Typhimurium

Overnight cultures of SL1344 were grown in the presence of LB (A, B), 7.5 mM $CuSO_4$ (C and D), 1.25 mM BCS (E**,** F) and 7.5 mM $CuSO_4$ and 1.25 mM BCS (G, H) at 37°C 200 rpm. Overnight cultures were stained with crystal violet and imaged at x100 oil immersion light microscopy. Data is a representative of three independent repeats performed in triplicate.

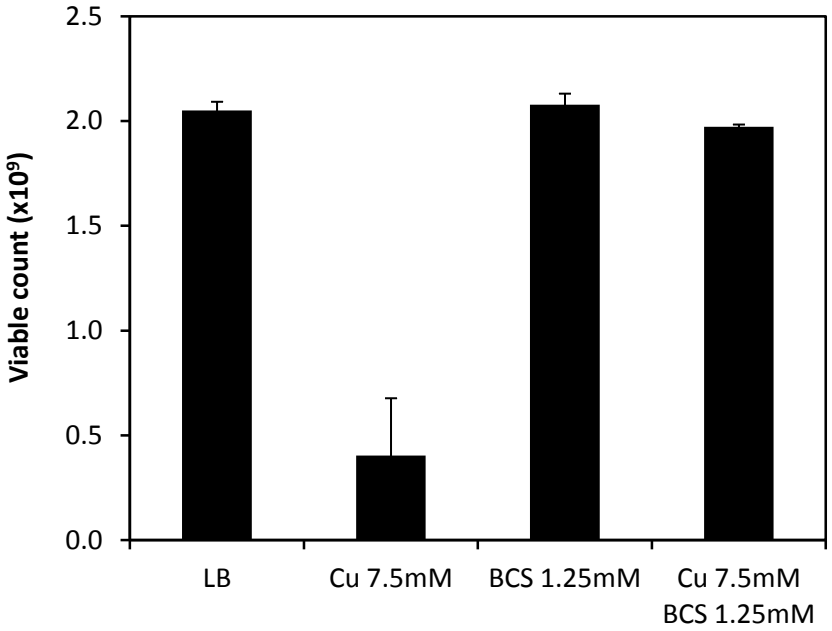


Figure 4.20 Copper toxicity toward S. Typhimurium is alleviated by BCS

Overnight cultures of SL1344 were grown in the presence of LB with and without supplementation of 7.5 mM $CuSO_4$ and 1.25 mM BCS as stated at 37°C 200 rpm. Bacteria was serially diluted in PBS and quantified by plating onto LB agar plates by the method of Miles and Misra. Data points represent the mean of three independent repeats performed in triplicate, error bars represent standard error.

4.9 Copper can induce the filamentation of S. Typhimurium with the *Salmonella* containing vacuole

It has been reported that *Salmonella* becomes filamentous during infection of macrophages (Rosenberger *et al.* 2004). Macrophages were seeded into wells containing sterilised cover slips and infections were performed, as previous. Macrophages were pre-treated with IFN-γ alone or with, L-NMMA and BCS. At time points coverslips were washed with PBS, fixed with paramforaldehyde, permeabilised with Triton-X and labelled with anti-O4 serum, anti-goat antibody and DAPI prior to examination by fluorescence microscopy (figure 4.20).

Filamentous S. Typhimurium were present internalised within IFN-γ activated macrophages confirming previous work that S. Typhimurium can become filamentous during intracellular infection (Rosenberger *et al.* 2004, Osman unpublished). Macrophages treated with BCS had substantially less filamentous S. Typhimurium within IFN-γ and IFN-γ + L-NMMA treated macrophages. The number of filamentous bacteria were quantified by counting internal S. Typhimurium within 100 random selected macrophages per repeat for each separate treatment. L-NMMA treated macrophages contained filamentous S. Typhimurium at a similar quantity to that of IFN-γ activated macrophages 32.83% (±2.10) compared to 35.50% (±3.73) respectively. This indicates that other agents are causing filamentation as well as reactive nitrogen species and that reactive nitrogen species do not initiate a strong filamentation response within the *Salmonella* containing vacuole. Macrophages treated with BCS had significantly reduced numbers of internalised filamentous S. Typhimurium than IFN-γ and IFN-γ + L-NMMA treated macrophages, 14.00% (±1.95). This identified that the copper antimicrobial response within macrophages is a factor contributing towards filamentation of S. Typhimurium.

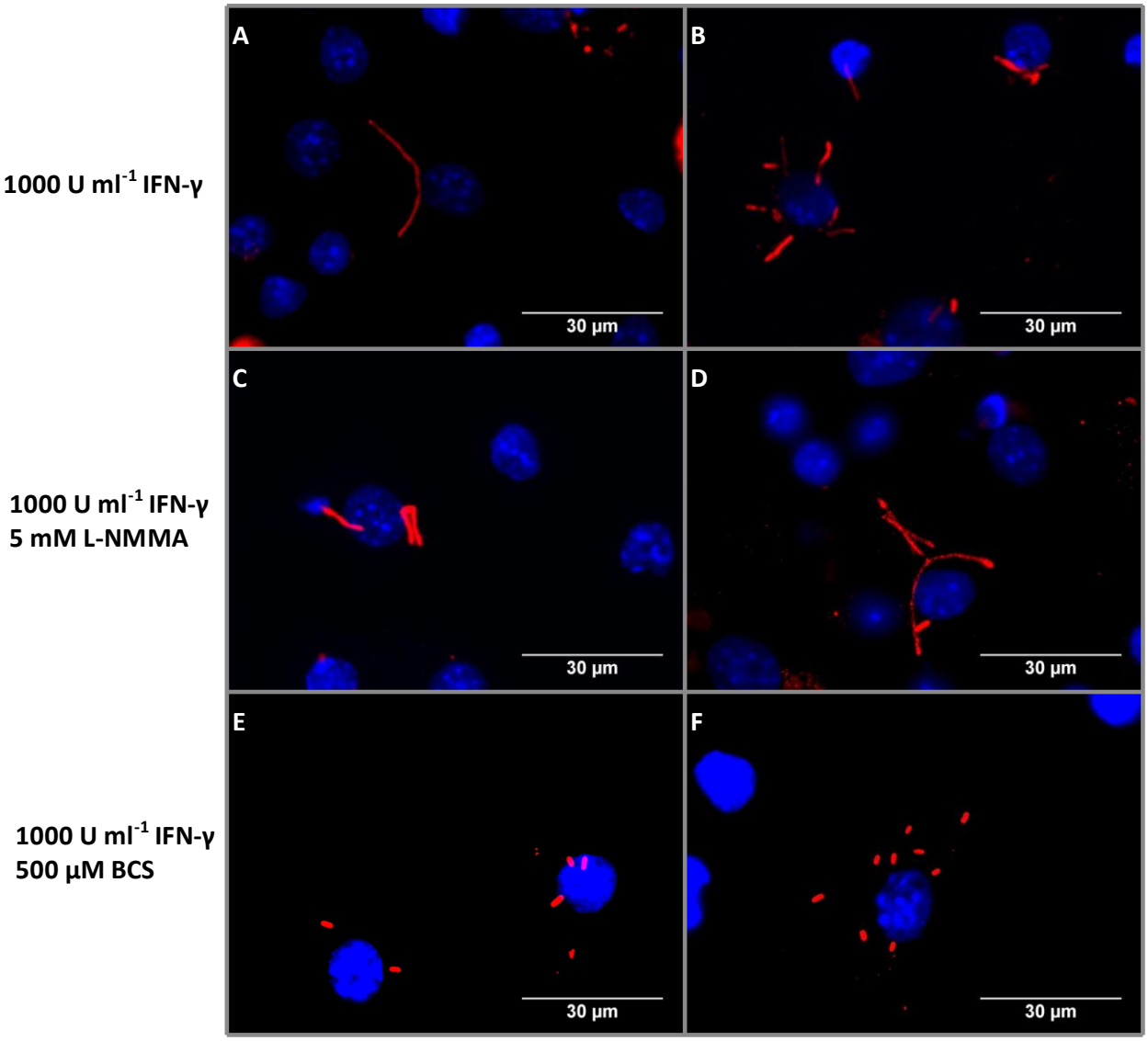


Figure 4.21 S. Typhimurium can become filamentous during infection of Raw 264.7 macrophages treated with IFN-γ alone or IFN-γ and L-NMMA but reduced filamentation is seen after IFN-γ and BCS treatment of macrophages

An overnight culture of SL1344 was infected into IFN-γ, IFN-γ and L-NMMA and IFN-γ and BCS treated macrophages at an MOI of 10:1 (bacteria:macrophages). At 24 hrs post infection coverslips were fixed and labelled with anti-O4 serum (red) and DAPI (blue). Images were visualised under x100 magnification. Data is a representative of three independent repeats performed in triplicate.

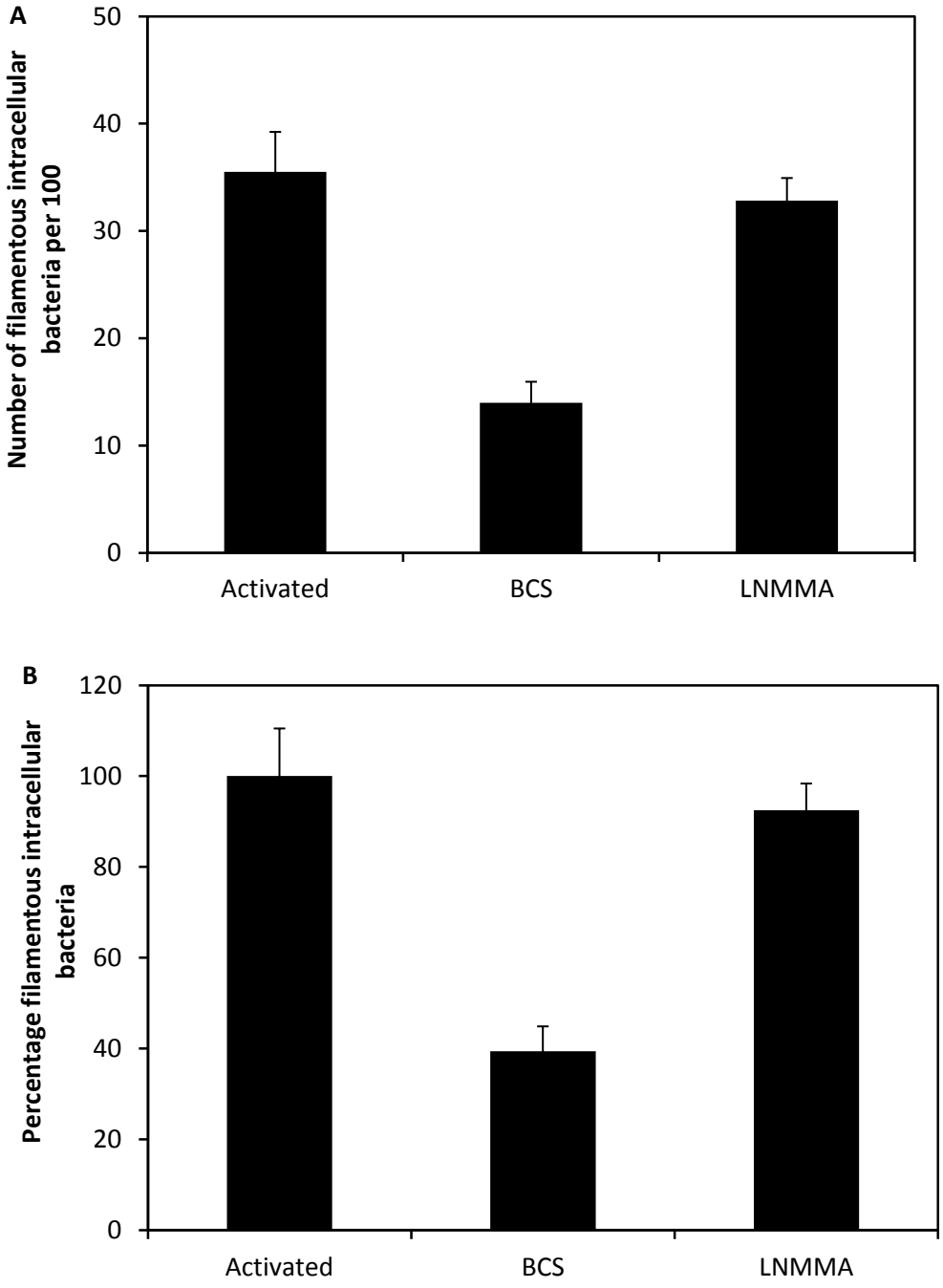


Figure 4.22 The addition of BCS reduces the number of filamentous intracellular S. Typhimurium

An overnight culture of SL1344 was used to infect Raw 264.7 macrophages at an MOI of 10:1 (bacteria : macrophage). At each time point macrophages were fixed and permeablised then incubated with anti-O4 serum washed then incubated with AlexaFluor 555 goat anti-rabbit antibody. (A) Filamentous bacteria within a random sample of 100 macrophage cells were quantified utilising a Nicon C1 microscope and (B) expressed as percentage of IFN-γ filamentous S. Typhimurium. Data points represent the mean of three independent repeats performed in triplicate, error bars represent standard error.

4.10 Investigating the importance of reactive oxygen species during infections of macrophages

It was identified by Osman *et al.* (2013) that CopA and GolT are required to supply copper to the copper chaperone CueP, which subsequently metallates $SodC_{II}$. The intracellular survival of *ΔcopA/ΔgolT.:cat* within macrophages could potentially stem from increased sensitivity to reactive oxygen species and was consequently investigated.

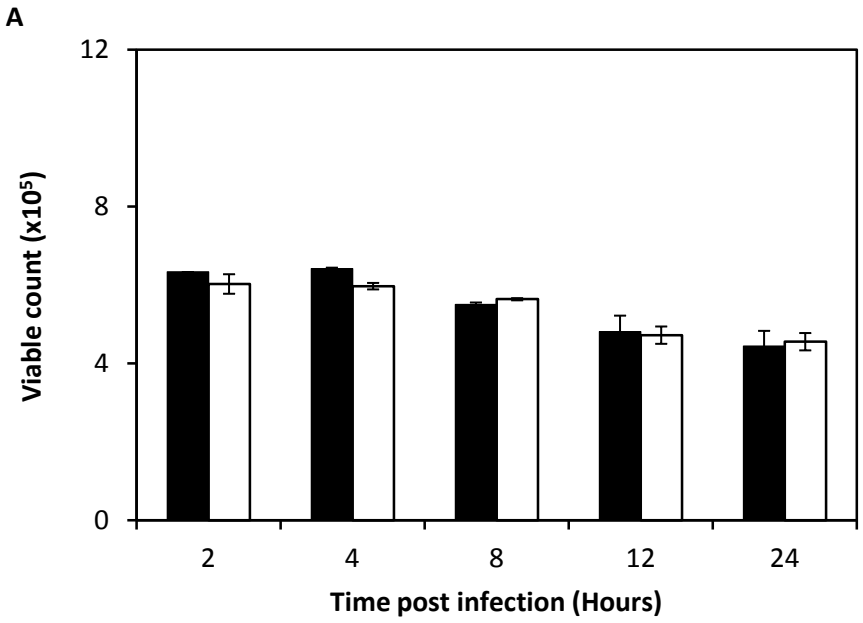
4.10.1 $SodC_{I}$ and $SodC_{II}$ do not contribute to S. Typhimurium virulence during infection of Raw 264.7 macrophages

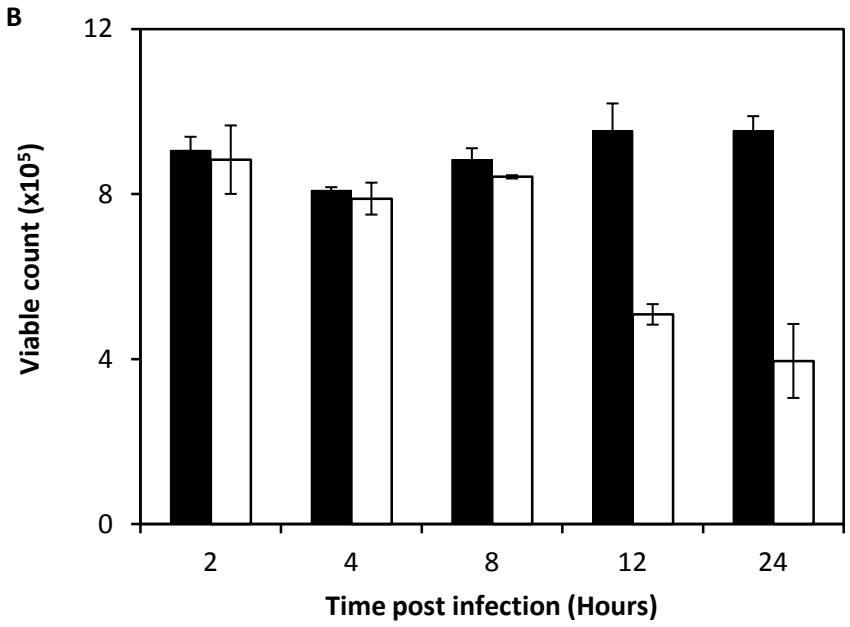
Superoxide dismutase enzymes detoxify superoxide and have been reported to aid S. Typhimurium virulence during intracellular infection of macrophages (Uzzau *et al.* 2002, Golubeva and Slauch 2006). The periplasmic Cu, Zn superoxide dismutase enzymes, $SodC_{I}$ and $SodC_{II}$, catalyse the conversion of superoxide into hydrogen peroxide. Competitive infections of IFN-γ activated macrophages were performed with SL1344 and *Δ$sodC_{I}$/Δ$sodC_{II}$.:cat* to examine the contribution of $SodC_{I}$ and $SodC_{II}$ to infections of Raw 264.7 macrophages under our infection conditions (figure 4.22). No difference in survival was observed between SL1344 and *Δ$sodC_{I}$/Δ$sodC_{II}$.:cat*; indicating that either Raw 264.7 macrophages do not produce a strong reactive oxygen species response or that S. Typhimurium is capable of detoxifying reactive oxygen species through its other two cytoplasmic superoxide dismutase enzymes, SodA and SodB, such that no phenotype is detectable. To further test the importance of $SodC_{I}$ and $SodC_{II}$ for infection of macrophages a quadruple mutant of *ΔcopA/ΔgolT/Δ$sodC_{I}$/Δ$sodC_{II}$.:cat* was created. As previously seen a *ΔcopA/ΔgolT.:cat* has increased killing compared to SL1344. No difference was seen between the level of survival between *ΔcopA/ΔgolT.:cat* and *ΔcopA/ΔgolT/Δ$sodC_{I}$/Δ$sodC_{II}$.:cat* further supporting that mutation of *$sodC_{I}$* and *$sodC_{II}$* does not affect S. Typhimurium virulence under these infection conditions (figure 4.22). These data imply that $SodC_{I}$ and $SodC_{II}$ do not provide a role for survival within Raw 264.7 macrophages and the reduced survival of *ΔcopA/ΔgolT.:cat* in macrophages is associated with the loss of copper resistance as opposed to loss of SodC activity.

To further test the role of $SodC_{I}$ and $SodC_{II}$ during infection of macrophages competitive infections were performed with bone marrow derived macrophages. Bone marrow derived macrophages provide a stronger antimicrobial response than immortalised cell line macrophages such as Raw 264.7. Progenitor bone marrow cells were extracted from the leg bones of C57BL/6 mice and differentiated in the presence of macrophage colony stimulating

factor. *ΔsodC$_I$/ΔsodC$_{II}$.:cat, ΔcopA/ΔgolT.:cat* and *ΔcopA/ΔgolT/ΔsodC$_I$/ΔsodC$_{II}$.:cat* were combined separately with SL1344 and used to infect bone marrow derived macrophages (figure 4.23).

As observed with Raw 264.7 macrophages all three mutant strains exhibited the same phenotypes during bone marrow derived competitive infections against SL1344. *ΔcopA/ΔgolT* had reduced survival at 12 and 24 hours post infection in comparison to SL1344. *ΔsodC$_I$/ΔsodC$_{II}$.:cat* exhibited no difference to SL1344 survival. *ΔcopA/ΔgolT/ΔsodC$_I$/ΔsodC$_{II}$.:cat* had reduced growth at 12 and 24 hours but no difference in survival to that of *ΔcopA/ΔgolT.:cat* indicating that SodC$_I$ and SodC$_{II}$ do not contribute to survival of S. Typhimurium under these conditions, whilst the ability to export copper via CopA and GolT does. Again this is consistent with reduced survival of *ΔcopA/ΔgolT.:cat* in macrophages being associated with a loss of copper resistance and hence increased vulnerability to elevated copper levels encountered in macrophage phagosomes.


A
12
8
4
0
Viable count (x10⁵)
2
4
8
12
24
Time post infection (Hours)


B
12
8
4
0
Viable count (x10⁵)
2
4
8
12
24
Time post infection (Hours)

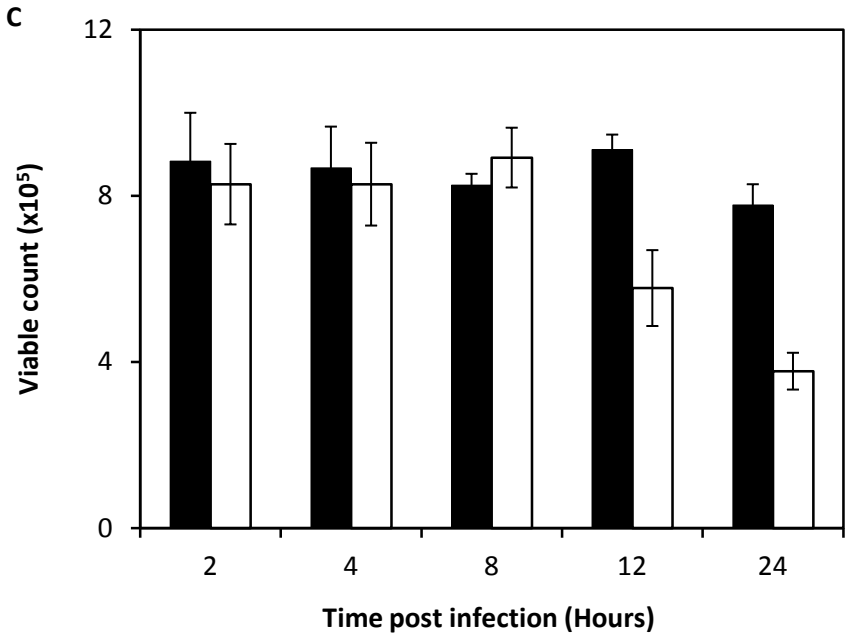
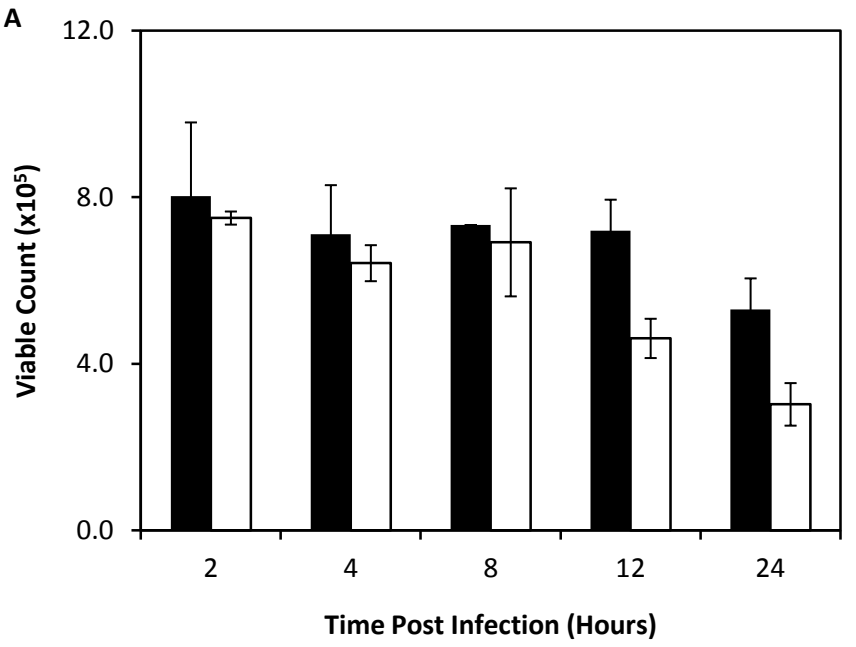
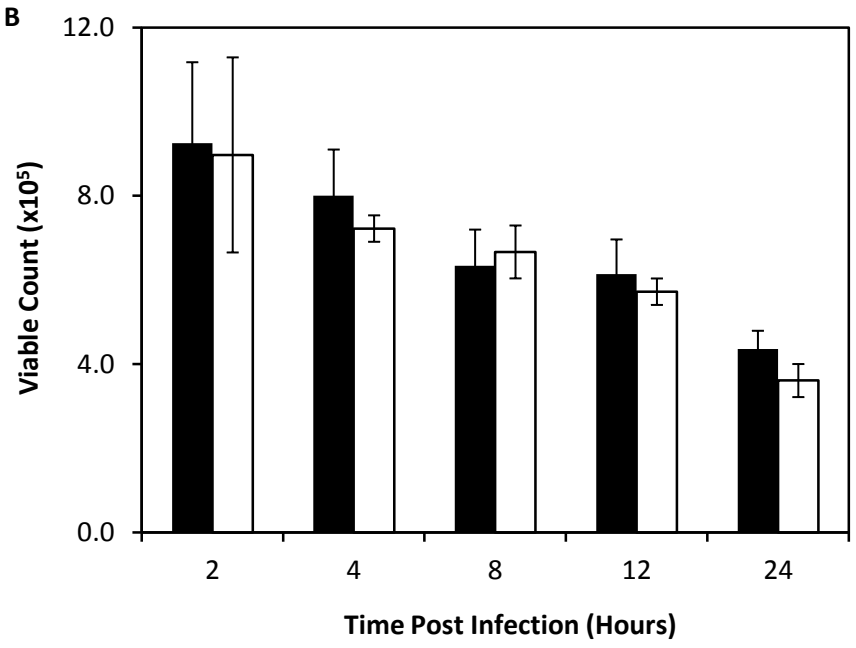


Figure 4.23 $SodC_I$ and $SodC_{II}$ do not influence S. Typhimurium viability during infection of Raw 264.7 macrophages

Competitive infections of SL1344 (black) and: (A) *ΔsodC$_I$/ΔsodC$_{II}$.:cat,* (B) *ΔcopA/ΔgolT.:cat* and (C) *ΔcopA/ΔgolT/ΔsodC$_I$/ΔsodC$_{II}$.:cat* (white), (mixed 1:1), within IFN-γ activated Raw 264.7 macrophages at an MOI of 10:1 (bacteria : macrophage). At indicated time points macrophages were lysed with 0.9% Triton-X and intracellular bacteria was serially diluted in PBS and quantified by plating onto LB agar plates with and without 10 µg ml^{-1} chloramphenicol to distinguish between SL1344 and *ΔcopA/ΔgolT.:cat* by the method of Miles and Misra, and grown at 37°C statically overnight. Data points represent the mean of three independent repeats performed in triplicate, error bars represent standard error.


A
12.0
8.0
4.0
0.0
Viable Count (x10^5)
2
4
8
12
24
Time Post Infection (Hours)


B
12.0
8.0
4.0
0.0
Viable Count (x10^5)
2
4
8
12
24
Time Post Infection (Hours)

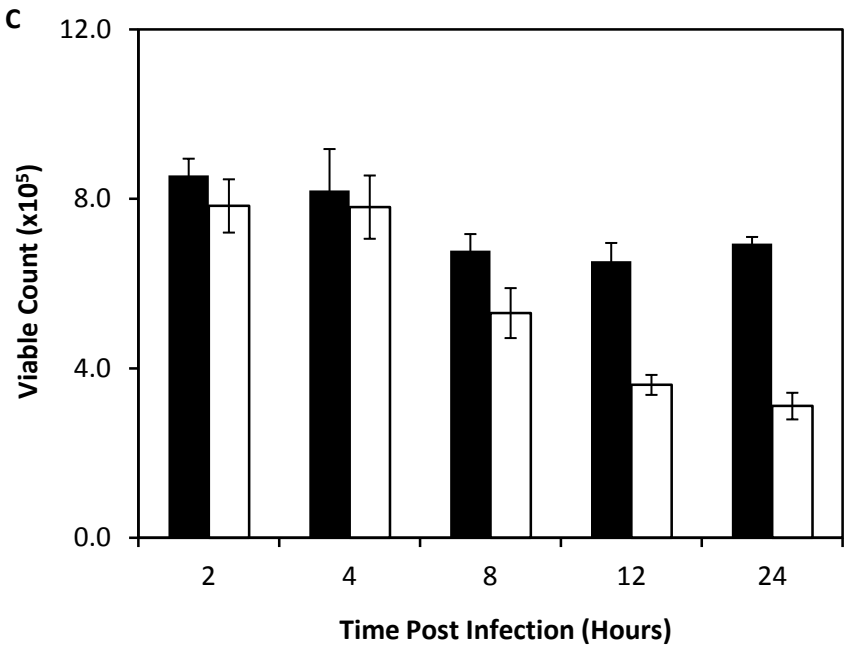


Figure 4.24 CopA and GolT contribute to S. Typhimurium virulence during infections of C57BL/6 bone marrow derived macrophages but $SodC_I$ and $SodC_{II}$ do not

Competitive infections of SL1344 (black) and (A) *ΔcopA/ΔgolT.:cat*, (B) *ΔsodC$_I$/ΔsodC$_{II}$.:cat* and (C) *ΔcopA/ΔgolT/ΔsodC$_I$/ΔsodC$_{II}$.:cat* (white), (mixed 1:1), within C57BL/6 bone marrow derived macrophages at an MOI of 10:1 (bacteria : macrophage). At indicated time points macrophages were lysed with 0.9% Triton-X and intracellular bacteria was serially diluted in PBS and quantified by plating onto LB agar plates with and without 10 µg ml^{-1} chloramphenicol to distinguish between SL1344 and *ΔsodC$_I$/ΔsodC$_{II}$.:cat, ΔcopA/ΔgolT.:cat* and *ΔcopA/ΔgolT/ΔsodC$_I$/ΔsodC$_{II}$.:cat* by the method of Miles and Misra, and grown at 37°C statically overnight. Data points represent the mean of two independent repeats performed in triplicate, error bars represent standard error.

4.10.2 CueP does not influence S. Typhimurium intracellular survival within Raw 264.7 macrophages

CueP has recently been identified as being required for supplying copper to $SodC_{II}$ (Osman *et al.* 2013). Whilst CueP does not appear to contribute to copper resistance of S. Typhimurium it is possible that CueP provides a mechanism to maintain $SodC_{II}$ activity whilst preventing deleterious side reactions of copper that may occur during infections due to copper-binding ability. Hence, the contribution of CueP to *S. typhimurim* survival in macrophages was investigated. Competitive infections of IFN-γ activated macrophages were performed with SL1344 and *ΔcueP.:cat* (figure 4.24). No difference in survival was present between SL1344 and *ΔcueP.:cat* identifying that CueP does not contribute to S. Typhimurium virulence during infection of Raw 264.7 macrophages. Furthermore, as CueP does not contribute to copper-resistance but does have a role in copper trafficking to $SodC_{II}$, these data provide further support for the reduced survival of *ΔcopA/ΔgolT.:cat* in macrophages being associated with the loss if copper resistance.

4.10.3 A *katG* mutant has similar survival to SL1344 during infection of Raw 264.7 macrophages

White *et al.* (2009) identified that increased copper with a macrophage could catalyse Fenton chemistry and increasing killing of intracellular bacteria within macrophages. However, by detoxifying hydrogen peroxide within the periplasm by the action of KatG, Fenton chemistry within the cytosol could be reduced. Hence, a *katG* mutant was investigated to identify if in its absence increased intracellular killing occurs. To investigate the importance of the periplasmic catalase KatG during an infection, a *katG* mutant strain was created. KatG catalyses the conversion of hydrogen peroxide into water and oxygen preventing oxidative damage to *Salmonella*. Competitive infections of IFN-γ activated Raw 264.7 macrophages were performed with SL1344 and *ΔkatG.:cat* (figure 4.25). No difference in survival was seen between SL1344 and *ΔkatG.:cat*. It is noteworhty that *Salmonella* possess two other catalase enzymes (KatEN) that could be functionally replacing KatG and preventing a detection of a phenotype associated with loss of KatG.

A triple mutant of *Δ$sodC_I$/Δ$sodC_{II}$/ΔkatG.:cat* was created to investigate if removing both periplasmic hydrogen peroxide and superoxide detoxification proteins would reduce *Salmonella* intracellular survival. Another triple mutant, *ΔcopA/ΔgolT/ΔkatG.:cat*, was created to identify if loss of *katG* in cells with increased cytoplasmic copper levels due to *copA* and *golT* mutation would result in increased Fenton chemistry. Competitive infections

were performed with SL1344 mixed at a 1:1 ratio with either *ΔsodC$_I$/ΔsodC$_{II}$/ΔkatG.:cat* or *ΔcopA/ΔgolT/ΔkatG.:cat* (figure 4.26). No difference was seen between SL1344 and *ΔsodC$_I$/ΔsodC$_{II}$/ΔkatG.:cat* survival. Having previously identified that *ΔsodC$_I$/ΔsodC$_{II}$* and *ΔkatG* do not exhibit any difference in survival to SL1344 possibly due to the presence of other enzymes capable of functionally replacing them no difference is seen when they are mutated within the same strain. The addition of a *katG* mutation does not alter the phenotype seen within *ΔcopA/ΔgolT.:cat* of reduced survival at 12 and 24 hours post infection compared to SL1344. It can be concluded that the mutation of *katG* alone is not enough to make S. Typhimurium sensitive to reactive oxygen species stress during infection of Raw 264.7 macrophages.

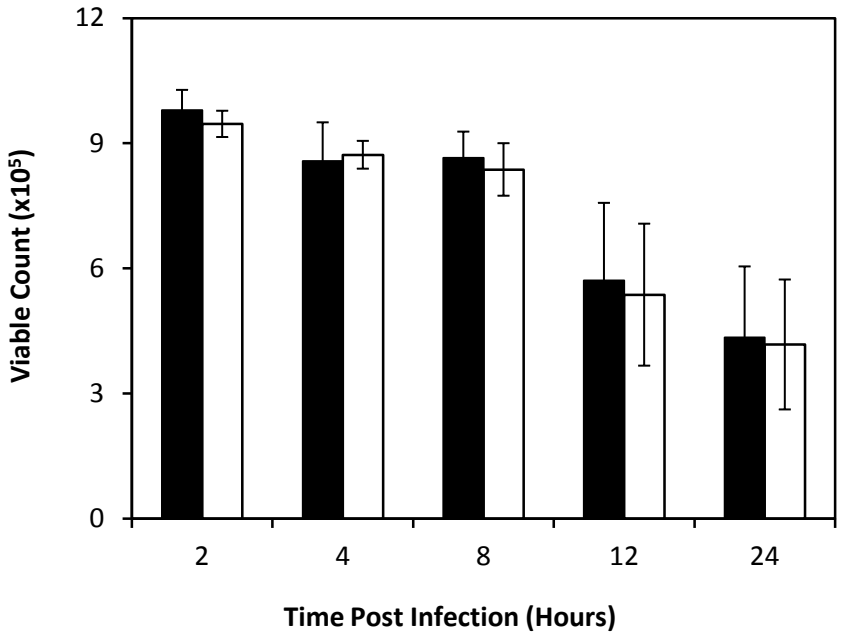
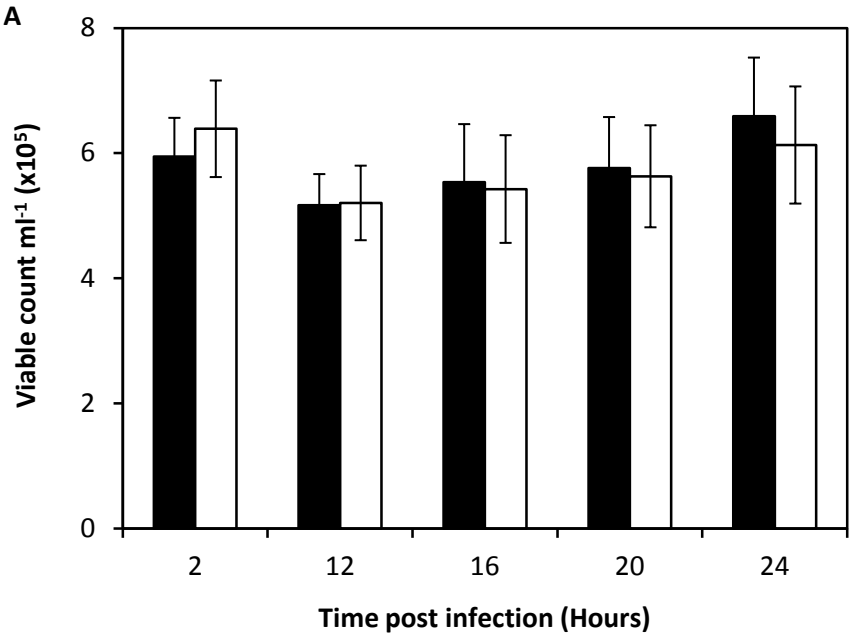
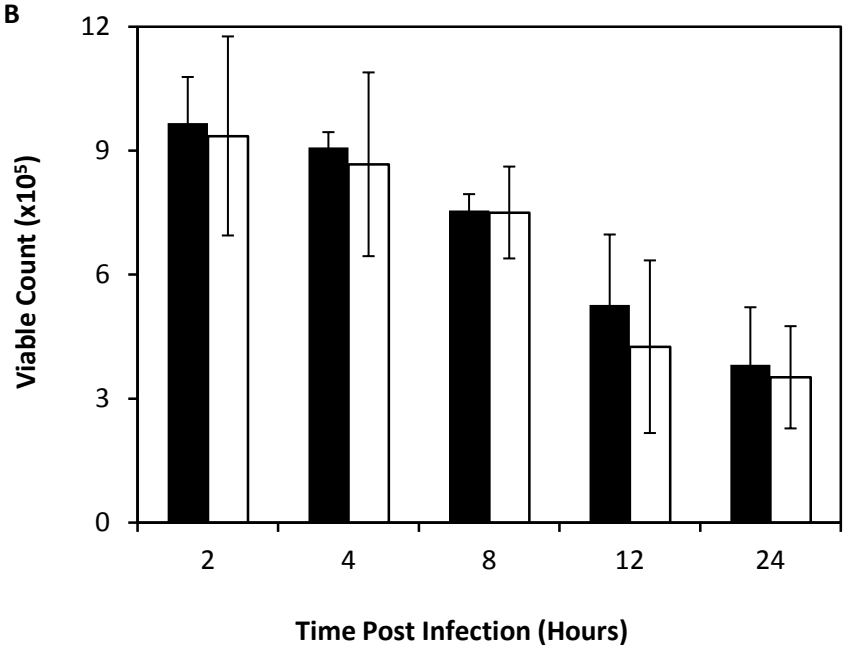


Figure 4.25 CueP does not contribute to S. Typhimurium virulence during infection of Raw 264.7 macrophages

Competitive infections of SL1344 (black) and *ΔcueP.:cat* (white), (mixed 1:1), within IFN-γ activated Raw 264.7 macrophages at an MOI of 10:1 (bacteria : macrophage). At indicated time points macrophages were lysed with 0.9% Triton-X and intracellular bacteria was serially diluted in PBS and quantified by plating onto LB agar plates with and without 10 µg ml^{-1} chloramphenicol to distinguish between SL1344 and *ΔcueP.:cat* by the method of Miles and Misra, and grown at 37°C statically overnight. Data points represent the mean of three independent repeats performed in triplicate, error bars represent standard error.


A
8
6
4
2
0
Viable count ml-1 (x105)
2
12
16
20
24
Time post infection (Hours)


B
12
9
6
3
0
Viable Count (x105)
2
4
8
12
24
Time Post Infection (Hours)

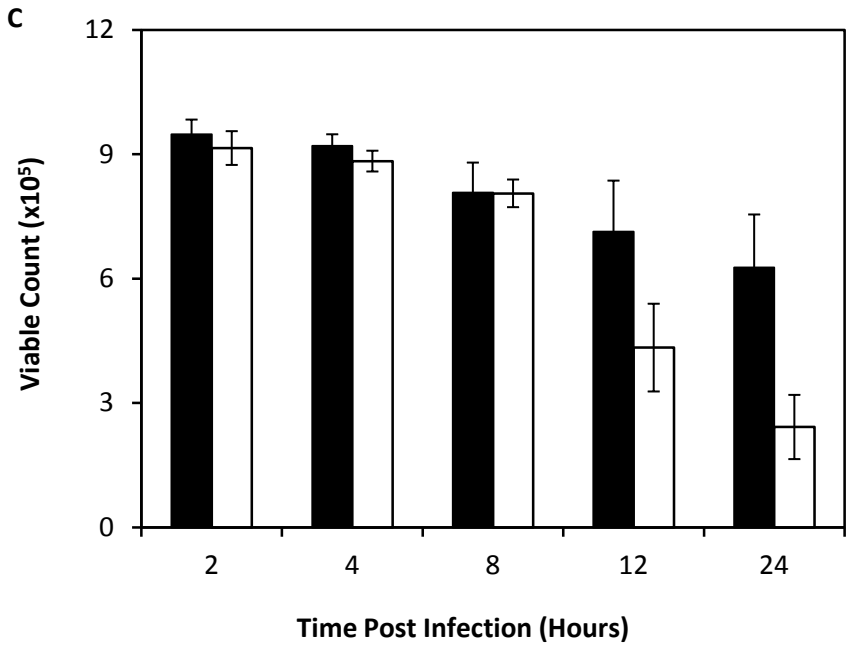


Figure 4.26 KatG does not affect S. Typhimurium survival in Raw 264.7 macrophages

Competitive infections of SL1344 (black) and (A) *ΔkatG.:cat* (white), (B) *ΔsodCI/ΔsodCII/ΔkatG.:cat* (white), (C) *ΔcopA/ΔgolT/ΔkatG.:cat* (white) and mixed 1:1 then infected within IFN-γ activated Raw 264.7 macrophages at an MOI of 10:1 (bacteria : macrophage). At indicated time points macrophages were lysed with 0.9% Triton-X and intracellular bacteria was serially diluted in PBS and quantified by plating onto LB agar plates with and without 10 µg ml^{-1} chloramphenicol to distinguish between SL1344 and *ΔkatG.:cat*, *ΔsodCI/ΔsodCII/ΔkatG.:cat* and *ΔcopA/ΔgolT/ΔkatG.:cat.:cat* by the method of Miles and Misra, and grown at 37°C statically overnight. Data points represent the mean of three independent repeats performed in triplicate, error bars represent standard error.

4.11 Discussion

4.11.1 S. Typhimurium is exposed to copper during infection of Raw 264.7 macrophages

Competitive infections of resting Raw 264.7 macrophages identified that *ΔcopA/ΔgolT.:cat* has reduced growth than SL1344 after 8 hours post infection, confirming previous work by Osman *et al.* (2010). Macrophages have been reported to utilise copper as an antimicrobial agent against intracellular pathogens, White *et al.* (2009). The double mutation of both *copA* and *golT* renders *Salmonella* highly susceptible to macrophage killing due to an inability to export copper from the cytoplasm. To test if S. Typhimurium copper export also provided a selective advantage in activated macrophages, competitive infections were performed using macrophages pre-treated with IFN-γ (figure 4.3). No replication was seen within IFN-γ activated macrophages for either SL1344 or *ΔcopA/ΔgolT.:cat*. Both strains exhibited a drop in viability over a 24 hour period of infection. Notably, *ΔcopA/ΔgolT.:cat* showed a significantly greater drop in viability than SL1344 after 8 hours post infection. Macrophages were pre-treated with IFN-γ to increase their killing ability against S. Typhimurium than non-IFN-γ treated macrophages (Varesio *et al.* 1984). Macrophages are known to increase synthesis of numerous antimicrobial compounds upon activation with IFN-γ (Gordon *et al.* 2005). Activation of macrophages by IFN-γ was supported by IL-1β ELISA detection assay confirming that activated mouse macrophages produce less IL-1β than non-activated, as previously indentified (Obermeier *et al.* 1999). The inability of S. Typhimurium to replicate and the drop in viability, combined with IL-1β levels detected confirmed the activation of macrophages. No large change was seen between the increased survival of SL1344 compared to *ΔcopA/ΔgolT.:cat* for resting and IFN-γ activated infections. A 2.8 fold difference in survival was present between SL1344 and *ΔcopA/ΔgolT.:cat* during infection of resting macrophages compared to a 2.9 fold difference during infection of IFN-γ activated macrophages. Competitive index values were also similar; 0.39 (±0.04during infections within resting macrophages and 0.35 (±0.05)) within IFN-γ activated macrophages. This suggests that IFN-γ activation does not increase the copper antimicrobial response within macrophages and requirement of S. Typhimurium to export copper in macrophages is unaffected by the activation state of macrophages. ICP-MS analysis of macrophages treated with IFN-γ compared to resting macrophages only identified a slight increase in copper levels of 14.10 (±0.44) x 10^3 atoms per cell compared to 12.48 (±0.74) x 10^3 atoms per cell. The ability of macrophage to prevent replication and begin to kill *Salmonella* was therefore not solely due to an increase in copper levels but other antimicrobial agents. Although a small change in copper quota was seen after activation by IFN-γ, IFN-γ may alter

mobilisation of a copper store within macrophages contributing to a copper antimicrobial response. White *et al.* (2009) identified an increase in macrophage copper uptake upon stimulation with either LPS or IFN-γ, of which only a small increase in copper accumulation was seen under this experimental protocol.

To test whether or not the reduced survival of *ΔcopA/ΔgolT.:cat* in comparison to SL1344 was associated with loss of both transporters as opposed to one or the other, *ΔcopA.:cat* and *ΔgolT.:cat* competitive infections using single mutants against SL1344 were performed within IFN-γ activated macrophages. No difference in viability was present for either *ΔcopA.:cat* or *ΔgolT.:cat* in comparison to SL1344 confirming the requirement for both copper transporters to be non-functional for reduced survival to be seen, consistent with known redundancy in function of CopA and GolT (Osman *et al.* 2010).

4.11.2 Reactive nitrogen species liberating copper from bound ligands within *Salmonella* is not the source of copper within the *Salmonella* containing vacuole during an infection

The difference in replication and survival between SL1344 and *ΔcopA/ΔgolT.:cat* occurs after 8 hours post infection. Previous work has identified that approximately after 8 hours post infection macrophages produce reactive nitrogen species (Eriksson *et al.* 2003). The production of nitrite was measured by the addition of DMEM from infection wells to Griess reagent. Nitrite levels increased from 12 hours post infection onwards, which correlates with the same time points that reduced survival of *ΔcopA/ΔgolT.:cat* is detected. The overlap in timing of when reactive nitrogen species are produced and the presence of copper within the *Salmonella* containing vacuole suggested that reactive nitrogen species may have a role in the release of copper. Previous experiments have suggested CopA and GolT provide protection against reactive nitrogen species (figure 3.10), with *ΔcopA/ΔgolT* having reduced growth in the presence of ASN, GSNO and NOC5/7. It has been identified that copper can initiate reactive species cycling with Cu^{+} capable of oxidising nitric oxide into a nitrosonium ion and also releasing a nitrosonium ion from a S-nitrosothiol group (Stamler *et al.* 1992, Singh *et al.* 1996). Therefore reduced survival of *ΔcopA/ΔgolT.:cat,* that contains high levels of intracellular copper, could be due to loss of reactive nitrogen species tolerance. Alternatively, Gold *et al.* (2008) identified that reactive nitrogen species such as nitric oxide can liberate copper from the metallothionine MymT within *M. tuberculosis*, which could be occurring within *Salmonella*. To examine the contribution of iNOS activity and hence reactive nitrogen species to increased killing of *ΔcopA/ΔgolT.:cat,* competitive infections were

performed in macrophages treated with the competitive inhibitor of iNOS (L-NMMA, Gibbs *et al.* 1987). Competitive infections within macrophages treated with IFN-γ and L-NMMA gave a significant difference ($p<0.05$) in survival between SL1344 and *ΔcopA/ΔgolT.:cat*. *ΔcopA/ΔgolT.:cat* had reduced viability from 12 hours post infection where as SL1344 replicated from 12 hours post infection onwards. This resulted in a larger fold difference between SL1344 and *ΔcopA/ΔgolT.:cat* of 11.06 in comparison to 2.9 for IFN-γ activated macrophages. The killing of *ΔcopA/ΔgolT.:cat* and inability to grow confirmed that reactive nitrogen species are not responsible for the reduced survival of *ΔcopA/ΔgolT.:cat* within a macrophage. The increase in survival of SL1344 confirmed previous work that iNOS is required to produce reactive nitrogen species within macrophages and reactive nitrogen species inhibiting replication of intracellular pathogens (Vazquez-Torres *et al.* 2000, Mastroeni *et al.* 2000).

Real-time PCR confirmed that even in the presence of L-NMMA expression of *copA* and *golT* was induced consistent with elevated levels of copper within *S. typhimurim* and a requirement for copper detoxification. No significant difference was present between the expression levels of *copA* and *golT* in *S. typhimurim* during infection of macrophages treated with IFN-γ and macrophages treated with both IFN-γ and L-NMMA. This corroborated viable count data that copper levels remain elevated in L-NMMA treated macrophages. ICP-MS analysis of macrophage copper levels identified no significant difference between L-NMMA and IFN-γ treated macrophages with 13.90 atoms per cell $x10^3$ compared to macrophages treated with IFN-γ alone, which had 14.09 atoms per cell $x10^3$.

4.11.3 The copper antimicrobial response within macrophages requires uptake of copper from the surrounding environment

If copper is not liberated from within *Salmonella* by reactive nitrogen species then it is likely to be transported to the *Salmonella* containing vacuole within the macrophage. Currently little is known about the storage of copper and the mechanism of distribution within macrophages. To determine the importance of uptaking copper from the surrounding environment macrophages were grown within DMEM containing 500 µM of the copper chelator BCS. BCS is not membrane permeable and binds to copper within DMEM inhibiting copper uptake by macrophages (Corson *et al.* 1997). ICP-MS analysis was performed upon macrophages grown in the presence of BCS and IFN-γ. Macrophages grown in BCS and pre-treated with IFN-γ contained 3.04 (±0.17) x 10^3 atoms per cell compared to 14.10 (±0.44) x 10^3 atoms per cell for IFN-γ treatment alone. The addition of BCS to DMEM before adding

to macrophages and activating with IFN-γ significantly lowered copper uptake. To test the effect of depriving macrophages of copper competitive infections were performed. Competitive infections of SL1344 and *ΔcopA/ΔgolT.:cat* within macrophages treated with BCS and IFN-γ gave no difference in viability between the strains. The treatment of BCS to DMEM increased the survival of *ΔcopA/ΔgolT.:cat* indicating that macrophages require an external source of copper to provide a copper antimicrobial response. Achard *et al.* (2012) also reported an increase in intracellular survival of S. Typhimurium during infection of macrophages treated with BCS.

To confirm that S. Typhimurium was exposed to reduced copper levels within *Salmonella* containing vacuole in BCS treated macrophages real-time PCR was performed to monitor *copA* and *golT* expression levels. Real-time PCR for *copA* and *golT* expression levels within SL1344 during macrophage infection treated with BCS and IFN-γ revealed significantly lower induction during an infection of IFN-γ activated macrophages. A difference of 9.27 and 12.98 gene fold change relative to *16s* and *rpoD* for *copA* and 5.02 and 6.65 gene fold change relative to *16s* and *rpoD* for *golT* was detected between IFN-γ and IFN-γ + BCS infections. This confirmed that macrophages uptake copper from the external environment and bactericidal activity is directly associated with copper availability. These data correlate with obtained by Achard *et al.* (2012) that identified macrophage expression of copper transporters Ctr1 and ATP-7a increase in response to S. Typhimurium infection of macrophages.

Future work could include infections performed with macrophages that are seeded into infection wells with DMEM and during an infection the DMEM is replaced with DMEM treated with BCS. This would identify if copper stores within macrophages are sufficient to kill *ΔcopA/ΔgolT.:cat* as occurs within IFN-γ activated macrophages. If reduced killing of *ΔcopA/ΔgolT.:cat* occurs this would suggest macrophages require a source of copper during an infection. Alternatively macrophages could be grown in DMEM treated with BCS providing macrophages with minimal copper then during an infection change to DMEM, providing a supply of copper during *Salmonella* infection. This would identify if macrophages can utilise a copper source directly from the environment to provide a copper antimicrobial response.

How *Salmonella* transports copper into the *Salmonella* containing vacuole is unknown. Achard *et al.* (2012) identified the presence of copper hot-spots upon S. Typhimurium infection of macrophages 12 hours post infection but they did not associate with the SCV. The formation of copper hot-spots timing corresponds with the timing of *ΔcopA/ΔgolT.:cat* reduced intracellular survival. White *et al.* (2009) first reported the use of copper as an antimicrobial agent against intracellular *E. coli* and identified the increased expression of

ATP-7a upon infection or activation of macrophages with either IFN-γ or LPS. White (*et al.* 2009) suggested the mechanism of copper transport is by ATP-7a binding to the phagosome and transporting copper from the cytosol into the phagosome. Osman (unpublished) and Achard *et al.* (2012) have both identified the increase of abundance for the copper transporter ATP-7a upon S. Typhimurium infection within a macrophage. Although Osman (unpublished) did not see an association of ATP-7a with the phagosome suggesting that ATP-7a does not bind to the *Salmonella* containing vacuole, co-localisation is seen between *E. coli* phagosome and ATP-7a, but *E. coli* and S. Typhimurium are diverse pathogens. A copper antimicrobial response is seen within 2 hours post infection against intracellular *E. coli* that corresponds within timings of the oxidative burst, whereas a copper antimicrobial response against intracellular S. Typhimurium is not seen until after 8 hours post infection (Osman *et al.* 2010, Achard *et al.* 2012). Competitive infection of an animal model of SL1344 and *ΔcopA/ΔgolT.:cat* results in reduced survival of *ΔcopA/ΔgolT.:cat*; but not in an animal model that has an ATP-7a deficient macrophage lineage (M. Petris, University of Missouri, personal communication). This indicates that ATP-7a is involved in supply of copper to the SCV but has yet to be identified how.

Salmonella inhibits the fusion of numerous antimicrobial agents with the *Salmonella* containing vacuole such as NADPH and iNOS to reduce exposure to both reactive oxygen and reactive nitrogen species (Vazquez-Torres *et al.* 2000, Chakavortty *et al.* 2002). It is possible that *Salmonella* is inhibiting the binding of ATP-7a to the *Salmonella* containing vacuole. Another possibility is that copper is transported within vesicles that fuse with the *Salmonella* containing vacuole. This would explain why copper is detected within the *Salmonella* containing vacuole but no association of ATP-7a is seen. Further research is required to identify the movement of copper within macrophages and to identify the mechanism by which copper is transported within the *Salmonella* containing vacuole.

4.11.4 Copper can induce filamentation of *Salmonella*

When *Salmonella* is exposed to an environmental stress it can induce filamentation, consequently individual bacteria can measure several µm in length. Filamentation typically occurs when the replication cycle is blocked within a bacterium. It has been reported that reactive nitrogen species and reactive oxygen species are capable of inducing filamentation (Schapiro *et al.* 2003, Rosenberger *et al.* 2004). Reactive nitrogen species can liberate zinc from DNA polymerase inhibiting DNA replication during cell division. Also, Richardson *et al.* (2012) identified nitric oxide induced-auxotrophy towards methionine, lysine and threonine

by S. Typhimurium upon exposure. Initially the ability of reactive nitrogen species to induce filamentation was investigated and confirmed. The reactive nitrogen species donors GSNO and NOC5/7 were both able to cause filamentation of overnight cultures of S. Typhimurium (figure 4.18) and overnight cultures of S. Typhimurium containing 7.5 mM $CuSO_4$ also exhibited filamentation (figure 4.19). When grown at this concentration of copper, bacterial growth was slightly inhibited with an overnight culture having a viable count of 4×10^8 compared to an untreated LB overnight having a viability of 2×10^9. Copper induced filamentation of bacteria has not previously been reported, although copper has been identified to encourage filamentation of the fungi *Cryptococcus neoformans* that is required for sexual replication (Lin *et al.* 2006).

It has been identified that S. Typhimurium can becomes filamentous during infection of macrophages with a role for reactive oxygen species and nitrogen species cited (Rosenberger *et al.* 2004, Osman unpublished). Having identified that copper can induce filamentation of S. Typhimurium it was investigated to identify if copper contributes to *Salmonella* filamentation within a macrophage. Infections were performed and intracellular S. Typhimurium were visualised by fluorescent microscopy. All macrophages were activated by pre-treatment with IFN-γ then additional supplementation included: no further supplementation, L-NMMA and BCS. IFN-γ activated macrophages, with no additional supplement resulted in the highest number of filamentous S. Typhimurium per 100 macrophages of 35.5. Henceforth 35.5 was used to calculate the percentage of filamentous S. Typhimurium from what was typically recorded. L-NMMA treatment was used to identify the involvement of reactive nitrogen species in causing filamentation. No significant difference to IFN-γ activated macrophages was seen for IFN-γ and L-NMMA treated macrophages, 92.48% of expected filamentous bacteria were seen in the absence of reactive nitrogen species. Macrophages treated with IFN-γ and BCS had significantly reduced numbers of filamentous bacteria. 39.44% of the expected filamentous bacteria were seen without the presence of an external copper source for the macrophages to utilise for a copper antimicrobial response. This identifies that copper can induce filamentation of S. Typhimurium within macrophages due to the significant difference in *Salmonella* filamentation ($p<0.05$) seen within IFN-γ pre-treated and IFN-γ and BCS treated macrophages. This supports that even SL1344 which has a functioning copper homeostatic system still incurs copper mediated stress within the *Salmonella* containing vacuole. Competitive infections with both S. Typhimurium SL1344 and *ΔcopA/ΔgolT.:cat* within IFN-γ and BCS treated macrophages had increased survival. The removal of the copper antimicrobial response by the addition of BCS aids SL1344 survival also supporting the notion that SL1344 experiences copper stress within the *Salmonella* containing vacuole.

Further work on this area would be to try and pinpoint the aspect of cell division that copper affects, starting with the SOS response initiator SulA, which is a key protein for initiating filamentation (Mukherjee *et al.* 1998). If a reporter construct to monitor expression from the promoter of *sulA* was used, promoter activity could be measured to determine whether or not copper increases transcription of *sulA*.

4.11.5 *ΔsodC$_I$/ΔsodC$_{II}$*, *ΔkatG* and *ΔcueP* do not exhibit any difference in survival to that of SL1344 during competitive infections of macrophages

Having confirmed the role of copper and reactive nitrogen species during *Salmonella* infection of macrophages, work was performed on the importance of reactive oxygen species detoxifying proteins. Superoxide dismutase enzymes SodC$_I$ and SodC$_{II}$ convert superoxide into hydrogen peroxide. SodC$_I$ has been reported to be important for S. Typhimurium virulence during infection of macrophages (Uzzau *et al.* 2002). Conflicting reports for the role of SodC$_{II}$ during macrophage infection have been reported (Golubeva and Slauch 2006, Krisnakumar *et al.* 2004). Osman *et al.* (2013) reported the requirement of either CopA or GolT for the transport of copper to CueP that subsequently transfers copper to SodC$_{II}$, identifying a link between copper homeostasis and reactive oxygen species detoxification.

No difference between SL1344 and *ΔsodC$_I$/ΔsodC$_{II}$.:cat* was seen for competitive infections performed with Raw 264.7 macrophages and bone marrow derived macrophages. It is unknown why *ΔsodC$_I$/ΔsodC$_{II}$.:cat* did not exhibit reduced survival within macrophages based on previous reports. Both mutations were verified by PCR (data not shown). *ΔsodC$_I$/ΔsodC$_{II}$.:cat* exhibited no sensitivity when exposed to reactive oxygen species such as hydrogen peroxide, paraquat and xanthine oxidase. The lack of sensitivity for *ΔsodC$_I$/ΔsodC$_{II}$.:cat* to reactive oxygen species has been reported, Craig and Slauch (2009), whom also did not see any difference in survival between S. Typhimurium 14028 and *ΔsodC$_I$/ΔsodC$_{II}$.:cat* when exposed to reactive oxygen species. The lack of sensitivity to reactive oxygen species is possibly due to the presence of two alternate superoxide dismutase enzymes (SodA, SodB). SodA and SodB provide a more prominent role in reactive oxygen species detoxification in S. Typhimurium during infection of macrophages than *SodC$_I$* and *SodC$_{II}$*, a *ΔsodA/ΔsodB.:cat* mutant is avirulent during an animal infection (Craig and Slauch 2009). Currently no alternate functions have been identified for *SodC$_I$* and *SodC$_{II}$* within *Salmonella*. If *ΔsodC$_I$/ΔsodC$_{II}$.:cat* does not exhibit sensitivity to reactive oxygen species it is not expected that *ΔsodC$_I$/ΔsodC$_{II}$.:cat* has reduced survival within

macrophages (Craig and Slauch 2009). A quadruple mutant of *copA/golT/sodC$_I$/sodC$_{II}$.:cat* was created to investigate if a phenotype was seen in the presence of excess cytoplasmic copper and removing supply of copper to SodC$_{II}$ (Osman *et al.* 2013). No difference in survival was seen for *ΔcopA/ΔgolT/ΔsodC$_I$/ΔsodC$_{II}$.:cat* compared to *ΔcopA/ΔgolT.:cat*. Hence, a mutation of *sodC$_I$* and *sodC$_{II}$* did not have an effect on survival of S. Typhimurium within both Raw 264.7 macrophages and bone marrow derived macrophages or in the presence of high intracellular complexed copper levels.

KatG is a periplasmic catalase that converts hydrogen peroxide into water and oxygen and is part of the reactive oxygen species detoxification system of *Salmonella*. Hydrogen peroxide is formed by superoxide dismutase enzymes and spontaneous production from electron leakage within the electron transport chain reducing water (Gonzalez-Flecha and Demple 1995). KatG is the only known periplasmic hydrogen peroxide degrading enzyme in *Salmonella* and its importance during intracellular survival was investigated due to its potential role in removing hydrogen peroxide before it can reach the cytosol and undergo Fenton chemistry. No difference in survival was seen between *ΔkatG.:cat* and SL1344 during competitive infections of Raw 264.7 macrophages pre-activated with IFN-γ and is consistent with previous work that also did not see reduced intracellular survival (Buchmeirer *et al.* 1995). Due to the presence of numerous enzymes capable of detoxifying hydrogen peroxide a greater number are required to be mutated or inhibited to affect reactive oxygen species resistance of S. Typhimurium. Hebrard *et al.* (2009) reported reduced intracellular survival upon deletion of all three catalase enzymes (*ΔkatG/ΔkatE/ΔkatN*), removing the functional redundancy still present in a *katG* mutant alone.

A *copA/golT/katG.:cat* triple mutant was created to test if the over accumulation of copper within the cytosol and increased potential for hydrogen peroxide to penetrate the cytosol would result in increased Fenton chemistry and reduced intracellular virulence. No significant difference was present between the survival of *ΔcopA/ΔgolT.:cat* and *ΔcopA/ΔgolT/ΔkatG.:cat* indicating the addition of a *katG* mutation does not alter reactive oxygen species resistance.

CueP has recently been identified to transfer copper to SodC$_{II}$ from the copper exporters CopA and GolT (Osman *et al.* 2013). The importance of CueP during macrophage infection was investigated. No difference in survival between *ΔcueP.:cat* and SL1344 during competitive infections of IFN-γ activated macrophages was seen. If CueP solely functions as a copper chaperone for SodC$_{II}$ then it would be expected to have the same survival as SL1344 based on a *sodC$_{II}$* mutant exhibiting no reduced survival within an infection model consistent with previous results (Golubeva and Slauch 2006, Craig and Slauch 2009).

4.11.6 Conclusions

The copper antimicrobial response S. Typhimurium encounters during intracellular survival within a macrophage is significantly reduced upon addition of the copper chelator BCS, as is expression of *copA* and *golT*. The timing of the copper antimicrobial response is not consistent with the oxidative burst. A *copA/golT* double mutant intracellular survival is unaffected by inhibition of iNOS and expression of *copA* and *golT* are similar to IFN-γ treated macrophages indicating copper toxicity is not directly linked to reactive oxygen or nitrogen species. Although the addition of copper to both reactive species can be additive through Fenton chemistry and sharing similar targets such as sulphur-iron clusters. The addition of BCS to macrophages significantly reduced the number of filamentous intracellular S. Typhimurium during macrophage infections highlighting a novel copper-antimicrobial phenotype within *Salmonella*.

Chapter 5

The role of TolC in copper homeostasis in S. Typhimurium

Salmonella possess two copper homeostasis systems, the *cue* and *gol* system. The *cue* systems encodes a P_{1B}-type ATPase (*copA*), a copper responsive transcription regulator (*cueR*), a multicopper oxidase (*cueO*) and a periplasmic copper binding protein (*cueP*). The *gol* system encodes a P_{1B}-type ATPase (*golT*), a copper responsive transcription regulator (*golS*) and a cytoplasmic copper binding protein (*golB*). Both systems are capable of exporting copper from the cytosol into the periplasm by the actions of CopA and GolT. A *copA/golT* double mutant accumulates approximately 25 fold greater copper levels than wildtype S. Typhimurium when grown at a sub-lethal copper concentration (Osman *et al.* 2010). This data implies the exportation of copper from the cytosol to the periplasm by CopA and GolT is important for the removal of copper from S. Typhimurium. Currently it is unknown how *Salmonella* removes copper from the periplasm into the external environment. Upon starting this study the periplasmic copper binding protein CueP had recently been discovered (Pontel *et al.* 2009, Osman *et al.* 2010). CueP is present in organisms lacking a *cus* system, such as is present in *E. coli* which is capable of exporting copper into the external environment. This suggested that CueP could be part of a copper detoxification system like the *cus* system that is capable of exporting copper outside of S. Typhimurium or functional substitute for the lack of a *cus* system (Pontel *et al.* 2009). As such, CueP could perform a similar function to the copper chaperone CusF that supplies copper from the periplasm to the CusCBA transport system for exportation (Kim *et al.* 2011). A gene cluster analysis was performed to analyse the genes located in proximity to *cueP*-like genes in other bacterial genomes in an attempt to identify potential outer membrane transporters that function in conjunction with CueP.

5.1 Gene cluster analysis using CueP

Gene clustering analysis was performed looking for genes encoding protein with sequence similarity to CueP within various bacterial species. A trend for gene encoding TolC dependent systems close to *cueP* was identified (figure 5.1). This highlighted that TolC and CueP may perform a role in copper tolerance for *Salmonella*.

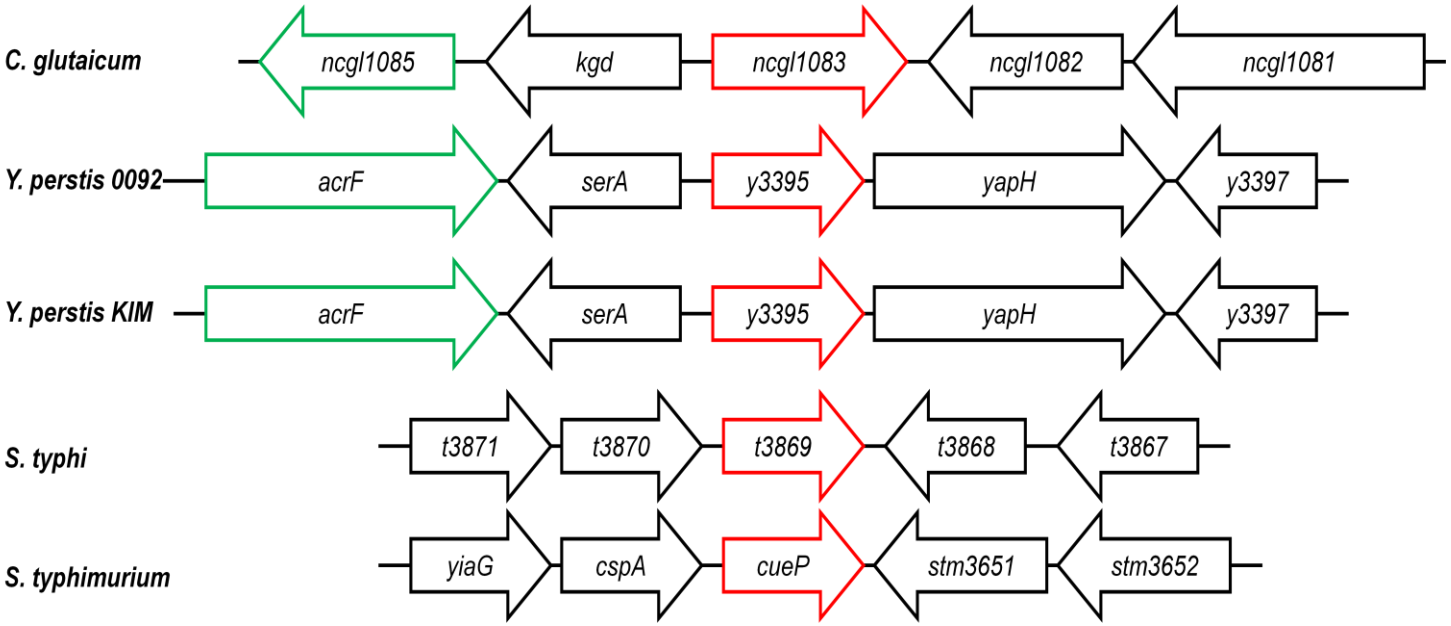


Figure 5.1 Clustering of TolC requiring systems within the vicinity of *cueP* sequence similarity genes

Genes encoding proteins with sequence similarity to CueP (outline red) cluster with *acrF* and *ncgl1085* (green outline) that recruit TolC as their outer membrane factor to remove substrates from within the organism to the external environment suggesting TolC may interact with CueP to provide copper tolerance.

5.2 Generation of a S. Typhimurium strain lacking *tolC*

It has previously been shown that TolC has a role in exporting several antimicrobial compounds and transition metals (Kim *et al.* 2011). Nishino *et al.* (2007) identified that a *tolC* mutant has reduced capability to grow under aerobic conditions in the presence of a variety of antimicrobial substrates including copper and zinc (Nishino *et al.* 2007). This did not identify if TolC provides a role in copper homeostasis or if a *tolC* mutant has increased susceptibility to copper mediated killing. This study aimed to examine the role of TolC in copper homeostasis and if TolC functions in conjunction with other known copper homeostasis proteins in S. Typhimurium.

A *tolC* mutant was initially created within S. Typhimurium strain LB5010a by insertional mutagenesis inserting a chloramphenicol resistance cassette into *tolC* resulting in the removal of the majority of *tolC* by homologous recombination as previously described (Datsenko and Wanner 2000). S. Typhimurium strain LB5010a does not contain the three chromosomal *Salmonella* DNA modification systems *hsdLT*, *hsdSA* and *hsdSB* that degrade foreign DNA, thus enabling the incorporation of the non-native chloramphenicol resistance cassette to create LB5010a *ΔtolC.:cat* (Bullas and Ryu 1983). The *tolC* mutation was subsequently transferred by P22 phage transduction into SL1344 strain of S. Typhimurium and checked by colony PCR to confirm transduction was successful (figure 5.2). The chloramphenicol resistance cassette was removed by the introduction of a flipase containing plasmid pCP20 into SL1344 *ΔtolC.:cat* and chloramphenicol sensitive colonies were screened by colony PCR to confirm the chloramphenicol cassette was removed confirming *ΔtolC* was created (figure 5.2). The resulting *ΔtolC* strains contained a scar sequence following the removal of the cat cassette. A complementation plasmid of pACYC184 *tolC* was also constructed by cloning the entire *tolC* gene including promoter and operator sequence into the multicopy plasmid pACYC184 and checked by sequencing of the plasmid. The pACYC184 tolC construct as well as pACYC184 empty vector were introduced into *ΔtolC* to generate the strains *ΔtolC* pACYC184 *tolC* and *ΔtolC* pACYC184, respectively

Table 5.1 Expected PCR product sizes for screening *tolC* deletion mutations

Genotype	Expected PCR product size (bp)
tolC	1760
ΔtolC.:cat	1374
ΔtolC.:scar	327

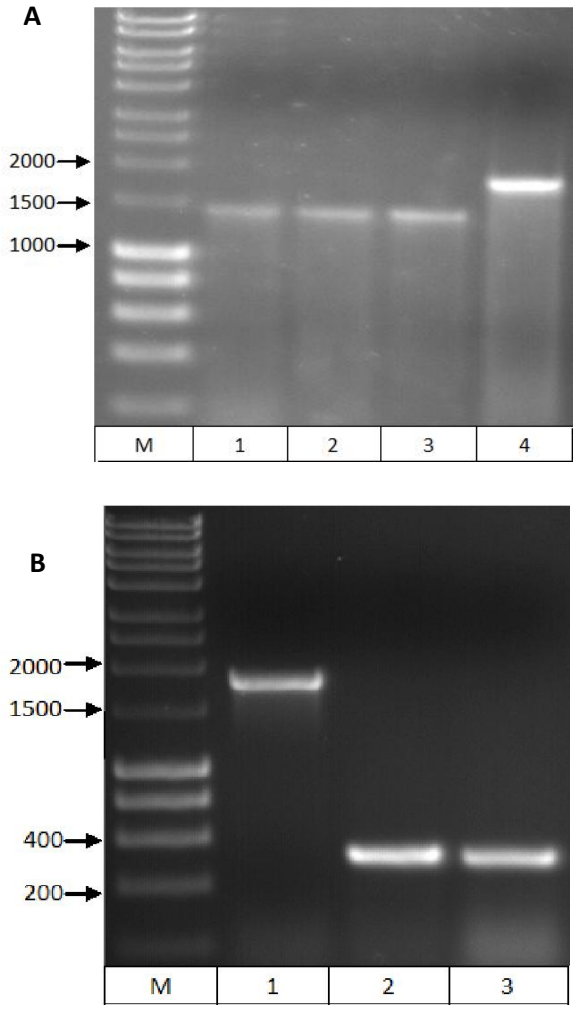


Figure 5.2 Confirmation of SL1344 *ΔtolC:.cat* and SL1344 *ΔtolC*:.*scar* strain creation

All numbered lanes contain products obtained using primers *tolC* _forward and *tolC*_ reverse in a PCR reaction. (A) Lanes 1 - 3 contained PCR products from colonies of LB5010a *ΔtolC.:cat* and lane 4 SL1344. (B) Lane 1 contained a colony of SL1344, lanes 2 and 3 contained colonies of SL1344 *ΔtolC*. After PCR reaction was completed agarose gel electrophoresis was performed with a 1% agarose gel. Lane M contained DNA hyperladder I of DNA markers of known molecular weight as indicated in base pairs.

5.3 Loss of *tolC* does not affect growth of S. Typhimurium in minimal media

TolC provides multiple functions as an outer membrane transporter but also contributes to maintaining stability of the outer membrane. Growth was monitored against time to generate a growth curve for SL1344 and *ΔtolC* to check whether or not a *tolC* mutant exhibits a change in growth rate. Overnight cultures of SL1344 and *ΔtolC* were diluted 1/100 into fresh minimal media and growth recorded until stationary phase was reached for both strains (figure 5.3). Log phase was reached by both strains after 2 hours, with cells entering stationary phase post 6 hours. No difference was seen in growth rate between SL1344 and *ΔtolC*, both exhibited an expected sigmoid growth curve, thus disruption of *tolC* does not affect growth in minimal media under aerobic conditions. Growth of the *tolC* mutant under metal stress could be analysed further.

5.4 TolC provides copper tolerance under aerobic conditions independent of CopA and GolT

5.4.1 TolC contributes to copper tolerance under aerobic growth conditions

To confirm the role of TolC in copper tolerance the growth of *ΔtolC* during copper stress was analysed. Endpoint copper tolerance growth assays were performed with SL1344, *ΔtolC*, *ΔtolC* pACYC184 and *ΔtolC* pACYC184 *tolC*. Overnight cultures were diluted 1/100 into fresh minimal media and grown in varying concentrations of $CuSO_4$ until early log phase (approximately 4-5 hours) and growth was quantified by measuring OD_{600} (figure 5.4). *ΔtolC* exhibited reduced growth at 25 µM $CuSO_4$ compared to SL1344, *ΔtolC* had an OD_{600} value of 0.16 (±0.03) and SL1344 0.25 (± 0.01). The reduced survival of the *ΔtolC* strain compared to SL1344 was also evident at increasing concentrations of copper with the largest difference occurring at 50 µM $CuSO_4$, *ΔtolC* OD_{600} 0.11 (±0.02) and SL1344 OD_{600} 0.23 (±0.01). A significant difference ($p<0.05$) was present between SL1344 and *ΔtolC* for; 25, 50, 75 and 100 µM $CuSO_4$. *ΔtolC* pACYC184 (containing the empty plasmid vector) exhibited similar OD_{600} readings to that of *ΔtolC* whereas *ΔtolC* pACYC184 *tolC* (containing the complementation plasmid) had similar copper tolerance to that of SL1344.

To further confirm that TolC contributes to copper tolerance under aerobic conditions, copper tolerance was monitored on copper-containing LB agar plates for SL1344, *ΔtolC*, *ΔtolC* pACYC184 and *ΔtolC* pACYC184 *tolC*. Overnight cultures were diluted by serial dilution to a concentration of 10^{-6} and the dilution ranges 10^{-6}-10^{-2} were plated onto LB agar containing varying concentrations of $CuSO_4$ and incubated overnight (figure 5.5). SL1344 and *ΔtolC*

pACYC184 *tolC* did not undergo any killing at the highest concentration of 4 mM $CuSO_4$, with full growth at the 10^{-6} dilution for both strains. *ΔtolC* and *ΔtolC* pACYC184 both had visible growth at 10^{-5} at 2 mM $CuSO_4$, a 1 log difference. At 3 mM $CuSO_4$ *ΔtolC* and *ΔtolC* pACYC184 had growth at 10^{-3} dilution. This equates to a three log difference in copper tolerance between a strain containing a *tolC* mutation and a strain possessing a functional *tolC* gene. The reduced growth of *ΔtolC* during endpoint assay and increased killing during copper-containing LB agar plate assays confirm TolC provides a role in copper tolerance under aerobic conditions. The restoration of *ΔtolC* copper tolerance by the addition of pACYC184 *tolC* complementation plasmid confirmed that the reduced copper tolerance is due to a mutation of *tolC*.

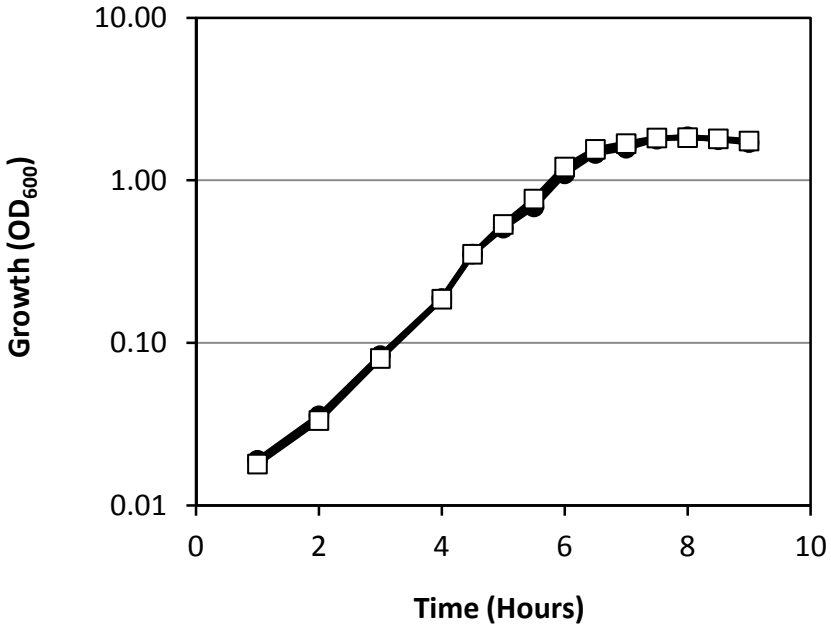


Figure 5.3 Loss of *tolC* does not influence S. Typhimurium growth rate in minimal media under aerobic conditions

An overnight culture of SL1344 (●) and *ΔtolC* (□) were diluted 1/100 into fresh minimal media and incubated at 37°C 200 rpm until stationary phase was reached, growth was determined by measuring OD_{600} every hour. Data points represent the mean of one repeat performed in triplicate, error bars represent standard error.

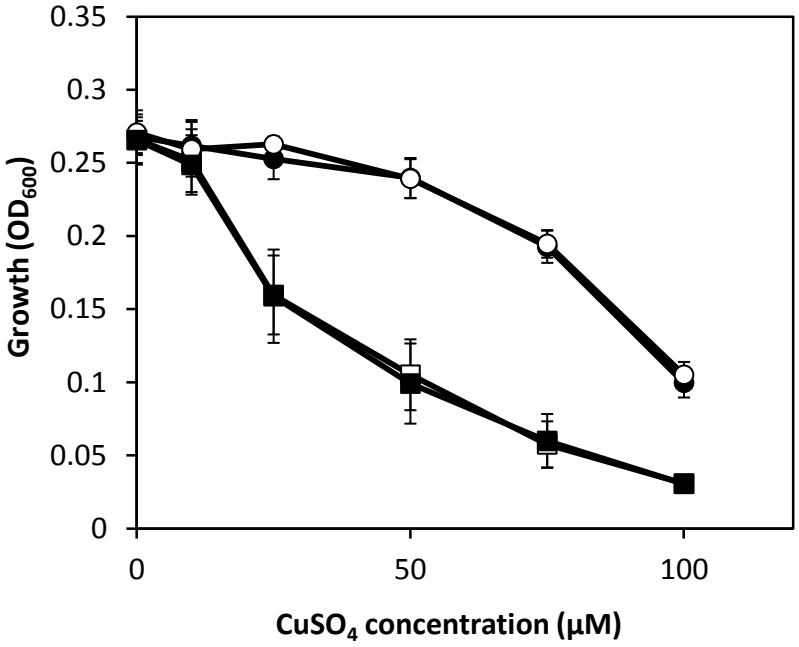


Figure 5.4 A S. Typhimurium *tolC* mutant has reduced tolerance to copper sulphate

Overnight cultures of SL1344 (●), *ΔtolC* (□), *ΔtolC* paCYC (■) and *ΔtolC* pACYC *tolC* (○) diluted 1/100 into fresh minimal media with varying $CuSO_4$ concentrations and incubated at 37°C, 200 rpm for 4-5 hours and growth measured at OD_{600}. Data points represent the mean of three independent repeats performed in triplicate, error bars represent standard error.

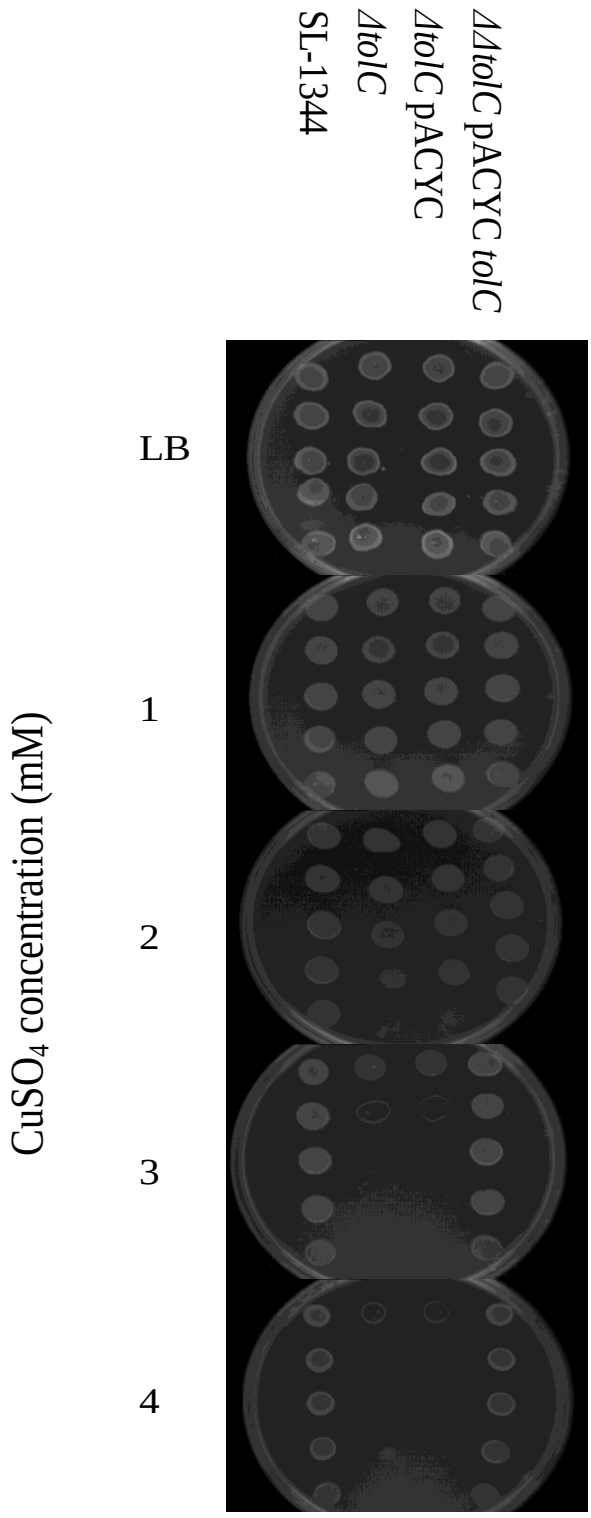


Figure 5.5 A *tolC* mutant has reduced copper tolerance when incubated on copper-containing LB agar plates

Overnight cultures of; SL1344, *ΔtolC*, *ΔtolC* pACYC 184 and *ΔtolC* pACYC 184 *tolC* were serially diluted from 10^{-2}–10^{-6} and plated onto LB agar plates containing varying $CuSO_4$ concentrations. Plates were grown statically at 37°C overnight and photographed. Data is a representative of three independent repeats.

5.4.2 A *copA/golT/tolC* triple mutant has decreased copper tolerance compared to a *copA/golT* double mutant

A *copA/golT* double mutant has reduced copper tolerance compared to wildtype (Osman *et al.* 2010). Having identified TolC performs a role in copper tolerance under aerobic conditions, an association between the roles of CopA, GolT and TolC was investigated. CopA and GolT could potentially associate with TolC to enable the export of copper from the cytosol to outside *Salmonella* or export copper into the periplasm for TolC to export outside of *Salmonella*. A *ΔcopA/ΔgolT/ΔtolC* triple mutant was created by phage transduction of *tolC* mutation into *ΔcopA/ΔgolT* strain. Endpoint copper tolerance growth assays were performed with SL1344, *ΔtolC*, *ΔcopA/ΔgolT* and *ΔcopA/ΔgolT/ΔtolC* (figure 5.6). Overnight cultures were diluted 1/100 into fresh minimal media and grown in varying concentrations of $CuSO_4$ until early log phase, growth was measured through absorbance OD_{600}. As shown in previous work by Osman *et al.* (2010) *ΔcopA/ΔgolT* has reduced growth at low copper concentrations. *ΔcopA/ΔgolT/ΔtolC* had reduced copper tolerance than *ΔcopA/ΔgolT* at 10 µM and 25 µM $CuSO_4$ concentrations. The greatest difference was present between *ΔcopA/ΔgolT* and *ΔcopA/ΔgolT/ΔtolC* was seen at 10 µM $CuSO_4$ with *ΔcopA/ΔgolT/ΔtolC* having an OD_{600} of 0.12 (± 0.03) and *ΔcopA/ΔgolT* an OD_{600} of 0.19 (± 0.05).

To further analyse copper tolerance of *ΔcopA/ΔgolT/ΔtolC* aerobic growth curves were performed with and without the presence of $CuSO_4$ (50 µM) in minimal media to monitor the growth rate of SL1344, *ΔtolC, ΔcopA/ΔgolT* and *ΔcopA/ΔgolT/ΔtolC*. Overnight cultures were diluted 1/100 into fresh minimal media and growth recorded until stationary phase was reached (figure 5.7). Cultures grown in the absence of copper exhibited no difference in growth rate with all strains reaching stationary phase after hour 10.

Growth curves in the presence of 50 µM $CuSO_4$ exhibited a difference in growth rate (figure 5.7). SL1344 growth rate was unaffected by the presence of copper indicating that copper levels up to 50 µM are non inhibitory to this strain. Adversely *ΔtolC, ΔcopA/ΔgolT* and *ΔcopA/ΔgolT/ΔtolC* had reduced growth in comparison to SL1344. *ΔtolC* had a slower growth rate than SL1344 but did obtain a similar final OD_{600} of 0.58 (± 0.01) compared to 0.63 (± 0.01) that SL1344 reached. *ΔcopA/ΔgolT* grew to a final OD_{600} of 0.10 (± 0.01). *ΔcopA/ΔgolT/ΔtolC* had the lowest final OD_{600} of 0.03 (± 0.01) indicating *ΔcopA/ΔgolT/ΔtolC* is unable to grow at a copper concentration of 50 µM. The combination of the endpoint and growth curve assays confirm that copper tolerance is lowered by the addition of a *tolC* mutation to a *copA/golT* double mutant. Both of these assays were performed within minimal media that has defined complexes within its composition giving increased copper availability compared to growth in complex media such as LB that contains a greater number of ligands

capable of complexing free copper. Copper tolerance was therefore also investigated in rich media to determine if the increase of complexed copper influences copper tolerance. Overnight cultures of SL1344, *ΔtolC, ΔcopA/ΔgolT* and *ΔcopA/ΔgolT/ΔtolC* were diluted by serial dilution to a concentration of 10^{-6}. Dilutions 10^{-6}-10^{-2} were plated onto LB agar containing varying concentrations of $CuSO_4$ and grown overnight; plates were photographed (figure 5.8). Both *ΔcopA/ΔgolT* and *ΔcopA/ΔgolT/ΔtolC* exhibited killing at 2 mM $CuSO_4$. Growth was present for *ΔcopA/ΔgolT* at 10^{-4} dilution whereas *ΔcopA/ΔgolT/ΔtolC* had growth at only the 10^{-2} dilution, a 2 log difference in survival. At 3 mM $CuSO_4$ no growth was seen for *ΔcopA/ΔgolT/ΔtolC* and *ΔcopA/ΔgolT* had growth in the final 10^{-2} dilution again showing reduced copper tolerance upon addition of a *tolC* mutation to a *copA/golT* double mutant. As seen previously a *tolC* mutant had reduced copper tolerance than SL1344. *ΔtolC* killing began at 3 mM $CuSO_4$ whereas SL1344 remained unaffected, the largest difference between the two strains occurred at 4 mM $CuSO_4$, SL1344 had viable bacteria in the 10^{-6} dilution whereas only *ΔtolC* had growth at 10^{-2} dilution, whilst *ΔcopA/ΔgolT* and *ΔcopA/ΔgolT*/*ΔtolC* where unable to grow.

The combined results for endpoint growth assay, growth curves and solid media copper tolerance assays all confirm that a *tolC* mutation is additive to a *copA/golT* double mutation in reducing copper tolerance. The additive nature of a *tolC* mutation to a *copA/golT* double mutation indicates that the mechanism of copper detoxification by TolC and that of CopA and GolT are separate. If the copper tolerance action of TolC was solely reliant upon the binding and transport of copper from the cytosol through either CopA or GolT no difference in copper toxicity would be expected between *ΔcopA/ΔgolT* and *ΔcopA/ΔgolT/ΔtolC*. Due to not knowing whether TolC receives copper from the cytosol from a cytoplasmic exporter or if copper is acquired in the periplasm by a membrane fusion protein several possibilities for the possible interaction of TolC and CopA/GolT, and is discussed further later (figure 5.21).

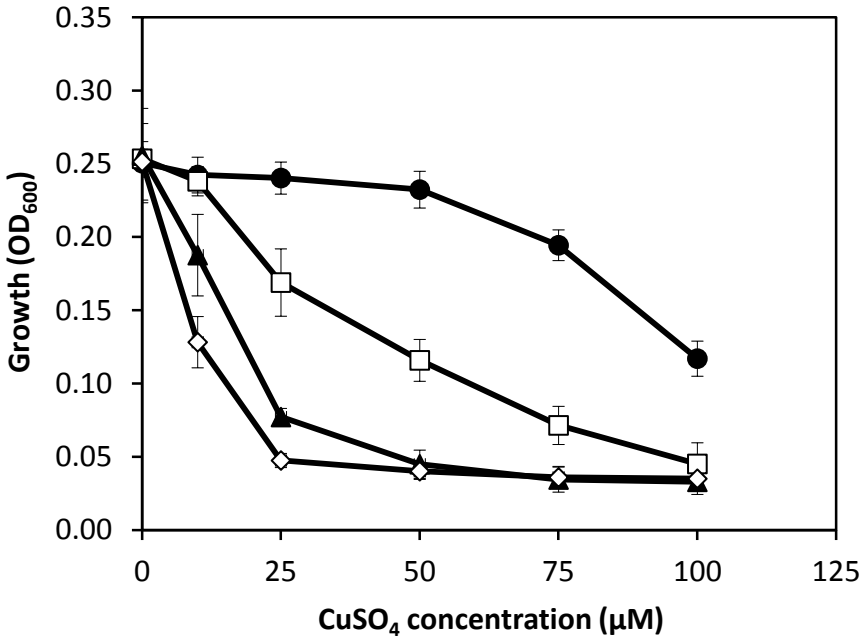


Figure 5.6 Loss of *tolC* gives reduced copper tolerance to a *copA/golT* double mutant

Overnight cultures of SL1344 (●), *ΔtolC* (□), *ΔcopA/ΔgolT* (▲) and *ΔcopA/ΔgolT/ΔtolC* (◊) diluted 1/100 into fresh minimal media at various $CuSO_4$ concentrations and incubated at 37°C, 200 rpm for 4 hours, growth was measured at OD_{600}. Data points represent the mean of three independent repeats performed in triplicate, error bars represent standard error.

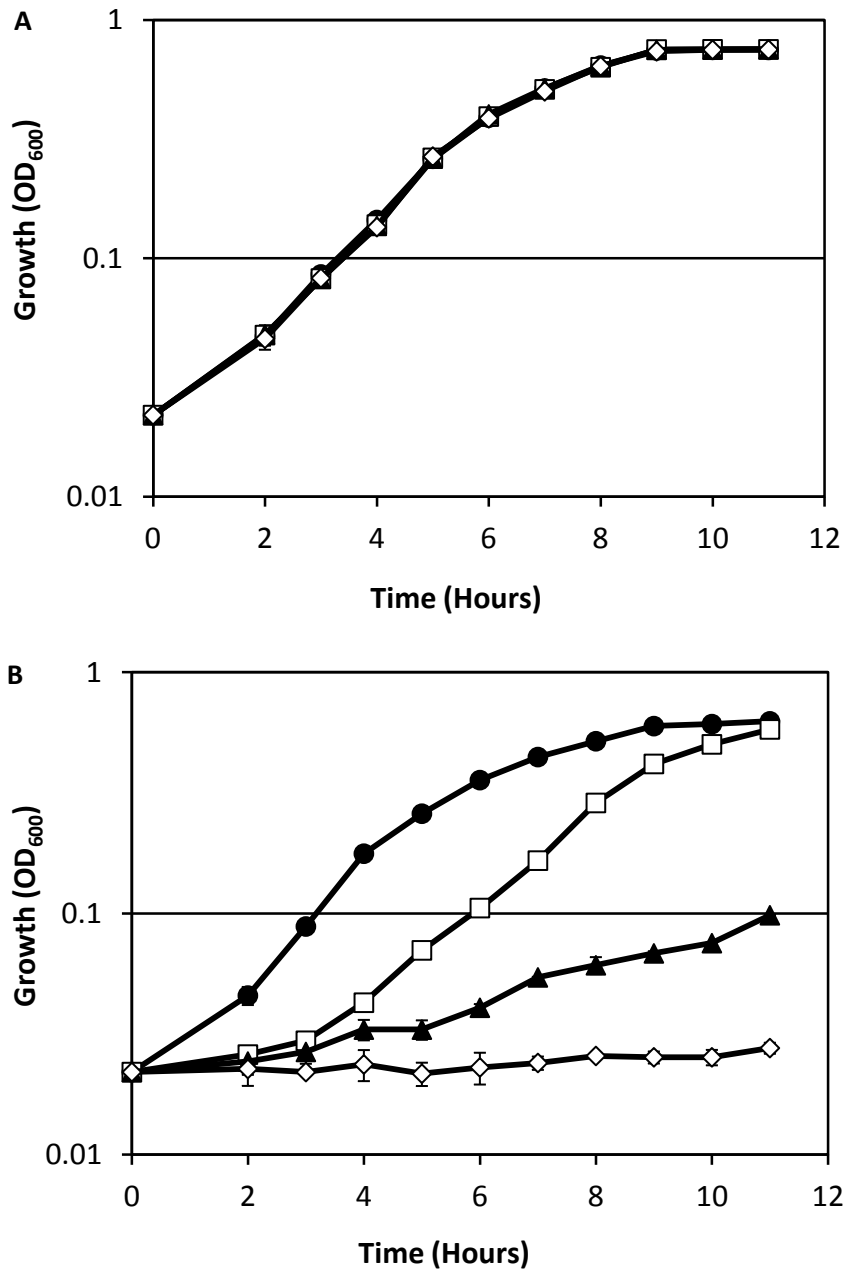


Figure 5.7 A *ΔcopA/ΔgolT/ΔtolC* mutant cannot grow in minimal media supplemented with 50 μM $CuSO_4$

Overnight cultures of SL1344 (●), *ΔtolC* (open square), *ΔcopA/ΔgolT* (▲) *and ΔcopA/ΔgolT/ΔtolC* (◊) diluted 1/100 into (A) fresh minimal media or (B) minimal media supplemented with 50 μM $CuSO_4$ and incubated at 37°C 200 rpm for 4-5 hours, growth measured at OD_{600}. Data points represent the mean of three independent repeats performed in triplicate, error bars represent standard error.

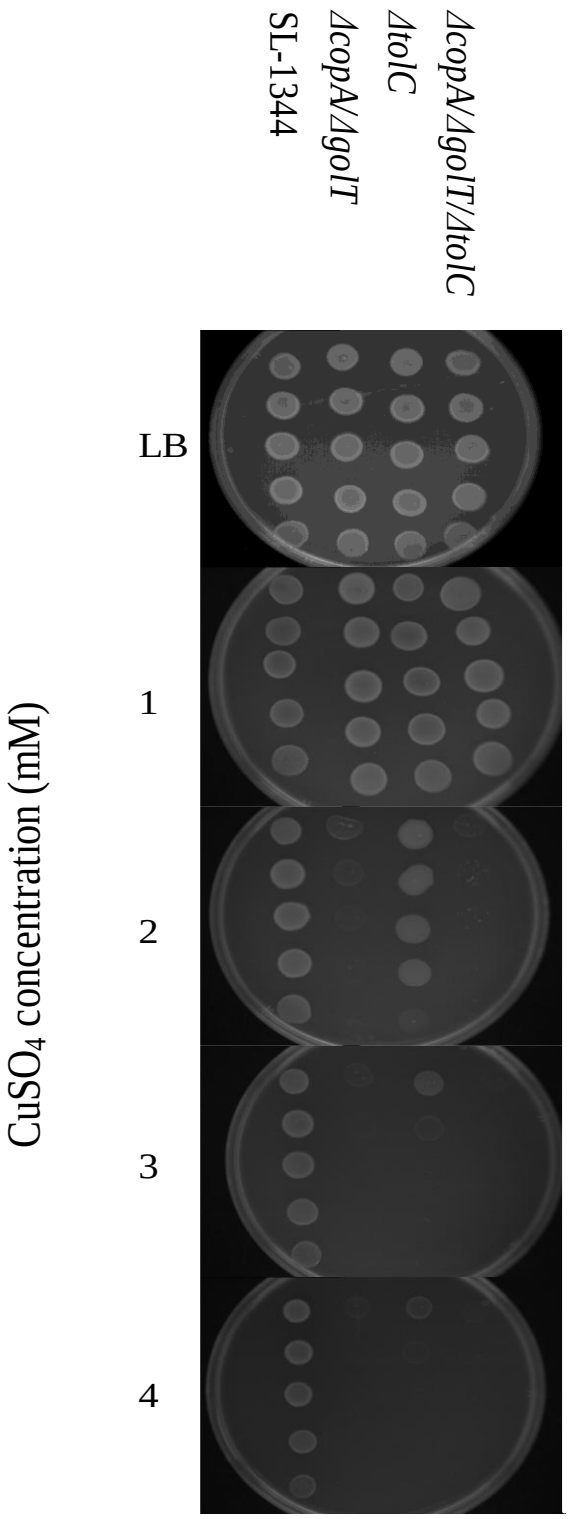


Figure 5.8 A *ΔtolC* mutation is additive to *ΔcopA/ ΔgolT* mutation

Overnight cultures of SL1344, *ΔcopA/ΔgolT*, *ΔtolC*, *ΔcopA/ΔgolT/ΔtolC* were serially diluted from 10^{-2}–10^{-6} plated onto LB agar plates containing varying $CuSO_4$ concentrations. Plates were incubated statically at 37°C overnight and imaged using a UV light transilluminator. Data is a representative of three independent repeats.

5.5 A *tolC* mutation affects copper contents within S. Typhimurium

5.5.1 A *tolC* mutant over-accumulates copper under non toxic copper sulphate concentrations

Having identified TolC contributes to aerobic copper tolerance under toxic copper concentrations the role of TolC during non-toxic copper concentrations was investigated. Intracellular levels of copper within a *tolC* mutant were analysed. An increased copper quota would indicate TolC has a role in copper homeostasis at low concentrations of copper rather than just at toxic copper concentrations. *O*vernight cultures of SL1344 and *ΔtolC* were grown in minimal media supplemented with 10 µM $CuSO_4$ (maximum permissive concentration). Bacteria were pelleted by centrifugation and washed with EDTA to remove external metals then lysed in nitric acid to release intracellular metals; samples were then analysed for metal content by ICP-MS. Data was presented as atoms per cell from viable counts of bacteria grown overnight, or as atoms per mg of protein quantified by Bradford assay of overnight bacteria. Data was expressed as both atoms per cell and atoms per mg of protein, to avoid discrepancy associated with a possible change in overall protein or cell morphology when *Salmonella* was stressed. It is estimated *Salmonella* contains less than one atom of free copper within the cytosol in the presence of copper homeostatic regulators CueR and GolS upregulating a copper homeostatic response above this level (Changela *et al.* 2003, Osman *et al.* 2013). Whilst *Salmonella* exposed to copper is anticipated to contain greater levels of copper than non-copper exposed cells bound or buffered within the cytosol to prevent free copper mediated damage. By expressing the data as atoms per cell and atoms per mg of protein it can be understood if a *tolC* mutant has a similar atoms per mg protein to SL1344 but a higher atom per cell value.

Analysis of intracellular copper levels of SL1344 and *ΔtolC* showed that in minimal media alone there was no difference in the total cellular copper contents. Only low levels of copper were detected in minimal media highlighting the limited amount of copper available (figure 5.9A). When minimal media was supplemented with 10 µM $CuSO_4$, *ΔtolC* had increased copper levels under non-toxic copper concentrations than that of SL1344 (figure 5.9B). *ΔtolC* accumulated more copper than SL1344 when expressed as both atoms per cell, 69.94 (± 8.76) compared to 14.15 (± 3.56) or when expressed as atoms per mg of protein, 129.10 (± 18.0) compared to 49.38 (± 6.60). A *tolC* mutation gave an increase of copper accumulation of 4.9 fold (atoms per cell) compared to 2.6 fold when expressed as atoms per mg protein. This confirmed that TolC performs copper homeostasis under non-lethal concentrations of copper ($p < 0.05$). The increase in intracellular copper levels indicated that *Salmonella* accumulate copper prior to exhibiting reduced growth. Copper homeostatic

proteins are upregulated in the presence of an abundance of copper prior to concentrations reaching a toxic level. The smaller fold change between SL1344 and Δ*tolC* for atoms per mg of protein than atoms per cell could relate to the upregulation of increased amounts of copper binding proteins such as GolB, CueP and CueO, due to the increased copper within Δ*tolC*.

5.5.2 A *tolC* mutation gives increased copper accumulation when combined with a *copA/golT* double mutation

Having identified a *tolC* mutation increased the copper quota of *Salmonella*, the copper quota of a *copA/golT/tolC* mutant was also investigated (table 5.2). Samples were prepared for ICP-MS as previously stated in the presence of 0.25 μM $CuSO_4$ due to *ΔcopA/ΔgolT/ΔtolC* having an extremely low tolerance to copper. A *copA/golT/tolC* triple mutant accumulated slightly more copper than a *copA/golT* double mutant when expressed as atoms per cell or atoms per mg of protein but the difference was not significant ($p = 0.17$). Previous work by Osman (*et al.* 2010) identified that a *copA/golT* double mutant accumulates significantly greater levels of copper to SL1344 and thus was also confirmed here. A *tolC* mutant accumulated more copper than SL1344 but was not a significant difference; this is possibly due to the copper concentration used being too low compared to previously (section 5.5.1). A *tolC* mutant accumulated a greater number of atoms per mg protein, 59.01 (± 3.57) $x10^3$ compared to 38.71 (± 14.50) $x10^3$ accumulated by SL1344. Hence, TolC appears to contribute to copper export at a higher copper concentration stimulus of 10 μM but does not play a significant role in copper export at low copper concentrations such as 0.25 μM. It can be concluded that CopA and GolT contribute to copper export at lower copper concentrations than TolC. If copper levels continue to rise, despite the copper detoxification actions of CopA and GolT, then TolC begins to provide copper detoxification to assist CopA and GolT to reduce the cellular copper load. The consistency of other divalent metals investigated between strains indicates no other divalent metals are exported by TolC, CopA or GolT. Cobalt was below the level of detection.

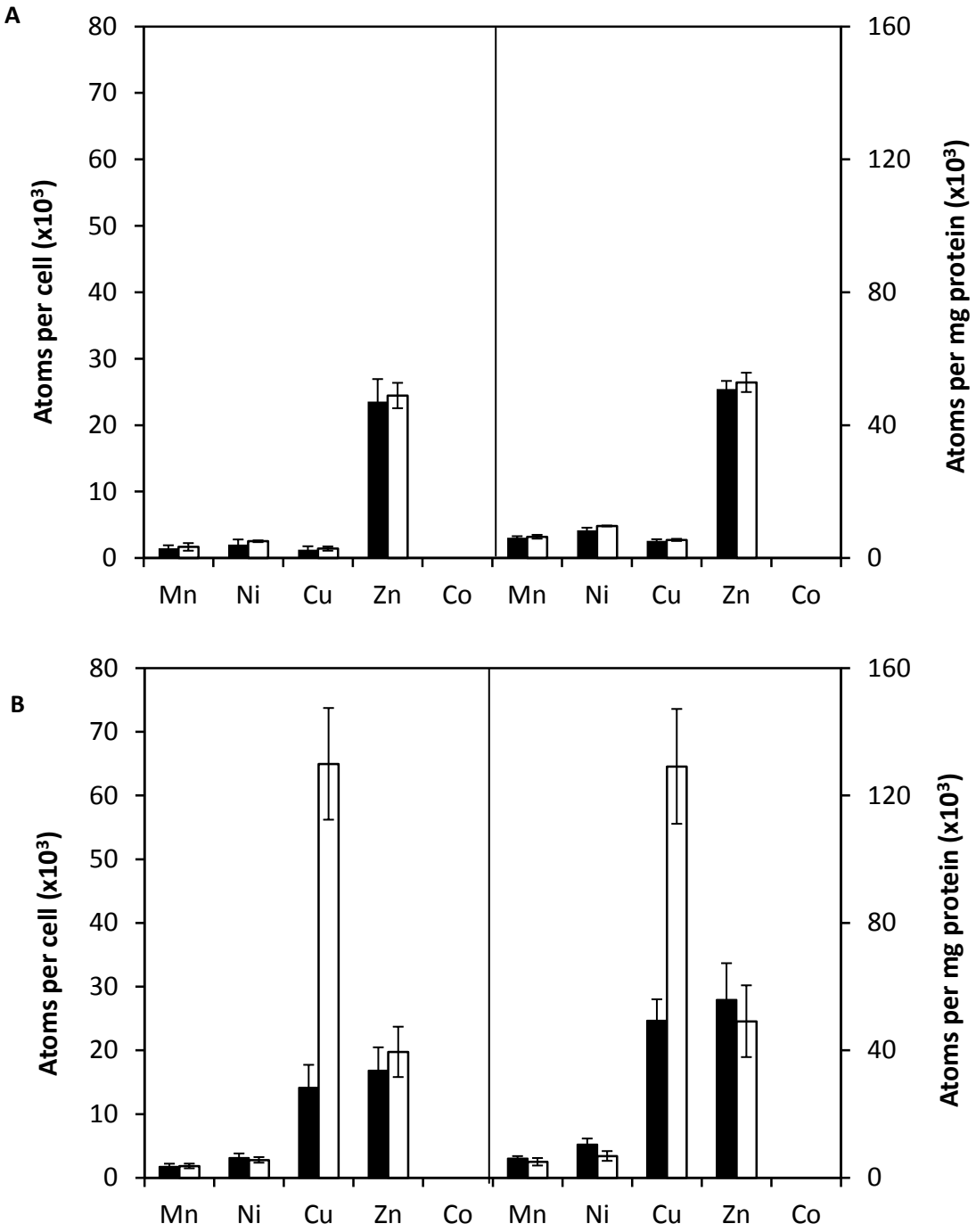


Figure 5.9 *ΔtolC* over accumulates copper at sub-lethal copper concentrations

Overnight cultures of SL1344 (black) and *ΔtolC* (white) in minimal media (A) or minimal media supplemented with 10 µM $CuSO_4$ (B) then washed with EDTA and lysed with nitric acid and intracellular metal levels quantified by ICP–MS analysis relative to number of atoms per cell (main axis) and number of atoms per mg of protein (secondary axis). Data points represent the mean of three independent repeats performed in triplicate, error bars represent standard error.

	Atoms per cell (x10^3)				
	Mn	Co	Ni	Cu	Zn
SL1344	2.13 (± 0.51)	0 (± 0.00)*	3.83 (± 1.00)	7.80 (± 1.89)	13.84 (± 3.38)
ΔtolC	1.92 (± 1 0.45)	0 (± 0.00)*	3.12 (± 0.70)	8.15 (± 2.89)	10.17 (± 4.56)
ΔcopA/ΔgolT	3.87 (± 1.00)	0 (± 0.00)*	4.85 (± 1.22)	229.37 (± 52.08)	23.15 (± 6.08)
ΔcopA/ΔgolT/ΔtolC	3.80 (± 1.03)	0 (± 0.00)*	3.31 (± 0.87)	292.49 (± 70.73)	23.85 (± 6.25)

	Atoms per mg protein (x10^3)				
	Mn	Co	Ni	Cu	Zn
SL1344	8.88 (± 0.79)	0 (± 0.00)*	17.19 (± 3.25)	38.71 (± 3.57)	87.44 (± 9.59)
ΔtolC	7.11 (± 0.34)	0 (± 0.00)*	13.93 (± 2.85)	59.01 (± 14.50)	70.29 (± 4.98)
ΔcopA/ΔgolT	13.07 (± 1.02)	0 (± 0.00)*	19.11 (± 6.69)	752.69 (± 304.12)	94.88 (± 10.85)
ΔcopA/ΔgolT/ΔtolC	12.12 (± 1.29)	0 (± 0.00)*	14.40 (± 1.54)	817.98 (± 331.65)	100.52 (± 13.45)

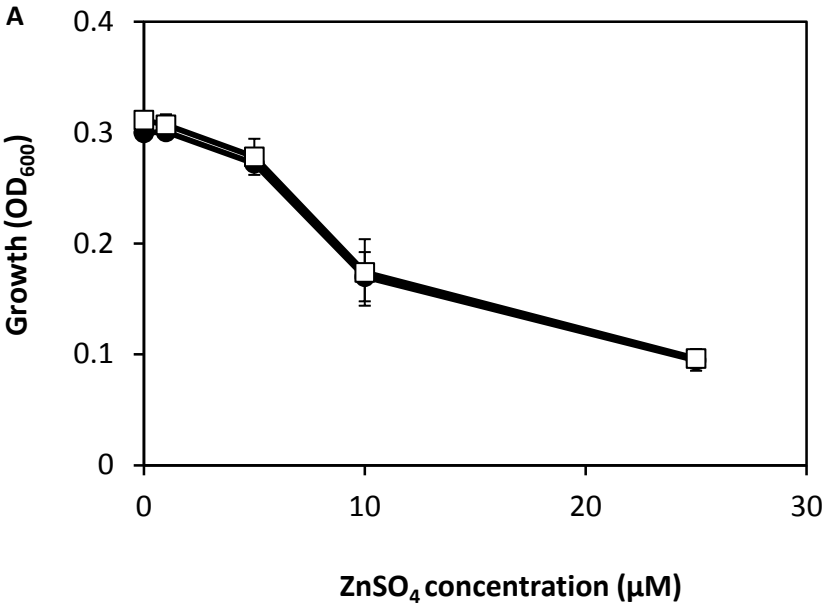
Table 5.2 *ΔcopA/ΔgolT/ΔtolC* accumulates greater amounts of copper than *ΔcopA/ΔgolT* when grown with 0.25 μM $CuSO_4$

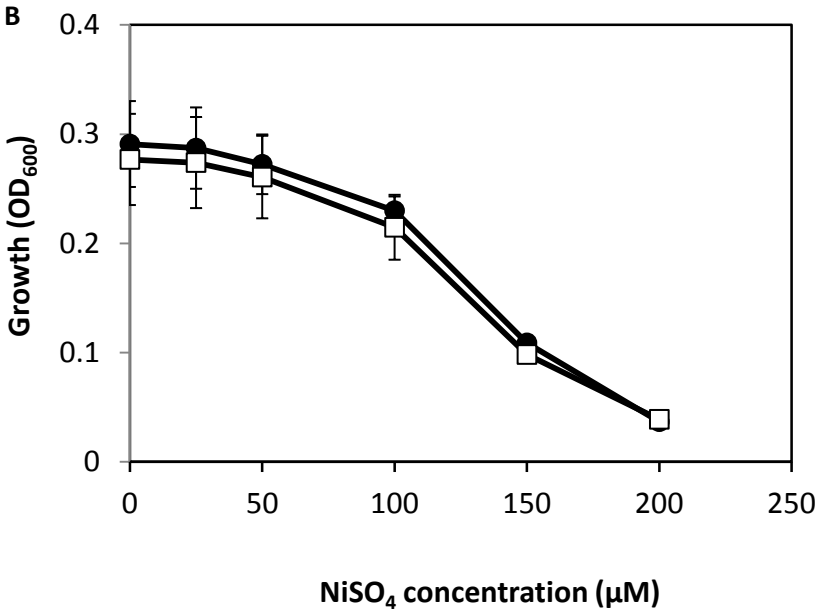
Aerobic overnight cultures of SL1344, *ΔtolC, ΔcopA/ΔgolT* and *ΔcopA/ΔgolT/ΔtolC* were grown in minimal media supplemented with 0.25 μM $CuSO_4$, washed with EDTA and lysed with nitric acid and intracellular metal levels quantified by ICP–MS analysis and calculated as number of atoms per cell (upper table) and number of atoms per mg of protein (lower table). Data is the mean of three independent repeats performed in triplicate, error bars represent standard error. *Cobalt below detectable limit.

5.6 TolC does not contribute to tolerance of zinc, nickel or cobalt in S. Typhimurium

Having confirmed TolC exports copper to provide a role in copper tolerance the tolerance of *ΔtolC* to other cation metals were investigated. It was previously reported by Nishino *et al.* (2007) that TolC is capable of exporting both copper and zinc under aerobic conditions. We have confirmed that TolC does export copper under aerobic conditions and therefore investigated other metal cations: zinc, nickel and cobalt. Endpoint metal tolerance growth assays were performed with SL1344 and *ΔtolC* (figure 5.10). Overnight cultures were diluted 1/100 into fresh minimal media and grown in varying concentrations of (A) $ZnSO_4$, (B) $NiSO_4$ and (C) $CoSO_4$ until early log phase and growth was measured by absorbance at OD_{600}. No difference in tolerance was seen for SL1344 and *ΔtolC* when exposed to; zinc, nickel or cobalt indicating that TolC does not export any of these metals under toxic concentrations.

To further analyse if TolC provides a role in the export of zinc, nickel and cobalt, ICP-MS analysis was performed to analyse metal content quotas under non-inhibitory concentrations (table 5.3). SL1344 and a *tolC* mutant had similar metal quotas for zinc, nickel and cobalt indicating that TolC does not provide a role in zinc, nickel or cobalt export at both inhibitory and non-inhibitory concentrations; indicating specificity towards copper export.


A
0.4
0.3
0.2
0.1
0
Growth (OD600)
0
10
20
30
ZnSO4 concentration (µM)


B
0.4
0.3
0.2
0.1
0
Growth (OD600)
0
50
100
150
200
250
NiSO4 concentration (µM)

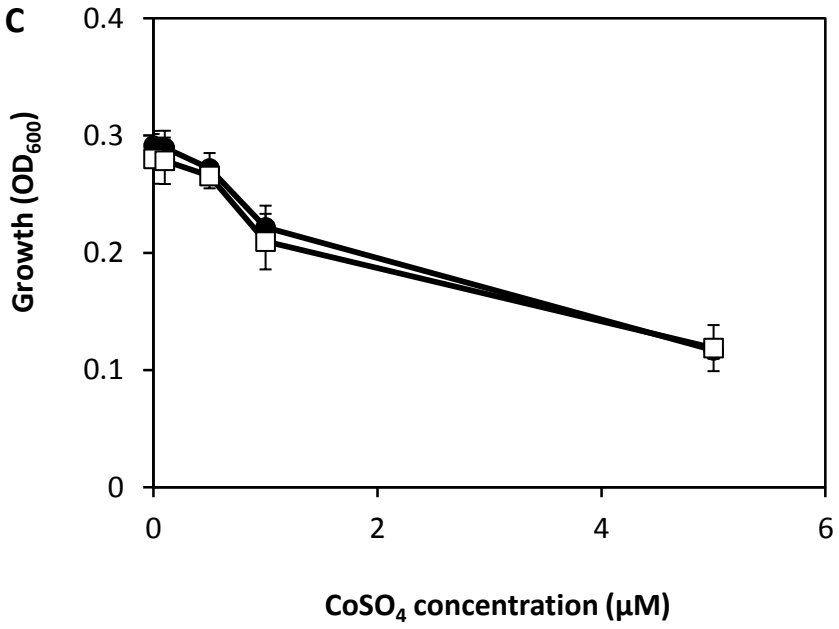


Figure 5.10 TolC does not contribute to tolerance to zinc, nickel or cobalt in S. Typhimurium

Overnight cultures of SL1344 (●), *ΔtolC* (□) diluted 1/100 into fresh minimal media with varying metal concentrations of (A) $ZnSO_4$, (B) $NiSO_4$ or (C) $CoSO_4$ and incubated at 37°C 200 rpm for 4-5 hours, growth measured at OD_{600}. Data points represent the mean of three independent repeats performed in triplicate, error bars represent standard error.

	Atoms per cell (x10^3)							
	Ni	25 μM Ni	Cu	10 μM Cu	Zn	5 μM Zn	Co	0.5 μM Co
SL1344	1.66 (± 0.78)	50.91 (± 3.81)	3.38 (± 2.74)	14.15 (± 3.56)	22.44 (± 16.03)	110.89 (± 15.05)	0.00 (± 0.00)	4.43 (± 1.87)
ΔtolC	1.91 (± 0.59)	50.87 (± 0.47)	5.23 (± 4.22)	64.94 (± 8.76)	24.94 (± 15.14)	109.80 (± 1.34)	0.00 (± 0.00)	4.67 (± 2.42)

	Atoms per mg protein (x10^3)							
	Ni	25 μM Ni	Cu	10 μM Cu	Zn	5 μM Zn	Co	0.5 μM Co
SL1344	9.94 (± 0.80)	112.70 (± 7.67)	15.27 (± 9.03)	49.38 (± 6.60)	111.56 (± 55.29)	516.58 (± 4.19)	0.11 (± 0.11)	12.69 (± 0.33)
ΔtolC	9.73 (± 0.97)	113.02 (± 4.07)	20.14 (± 12.58)	129.1 (± 18.02)	113.24 (± 48.57)	523.58 (± 21.63)	0.12 (± 0.12)	12.53 (± 3.16)

Table 5.3 At sub-lethal concentrations of divalent cations a *tolC* mutant over accumulates copper but not nickel, zinc or cobalt

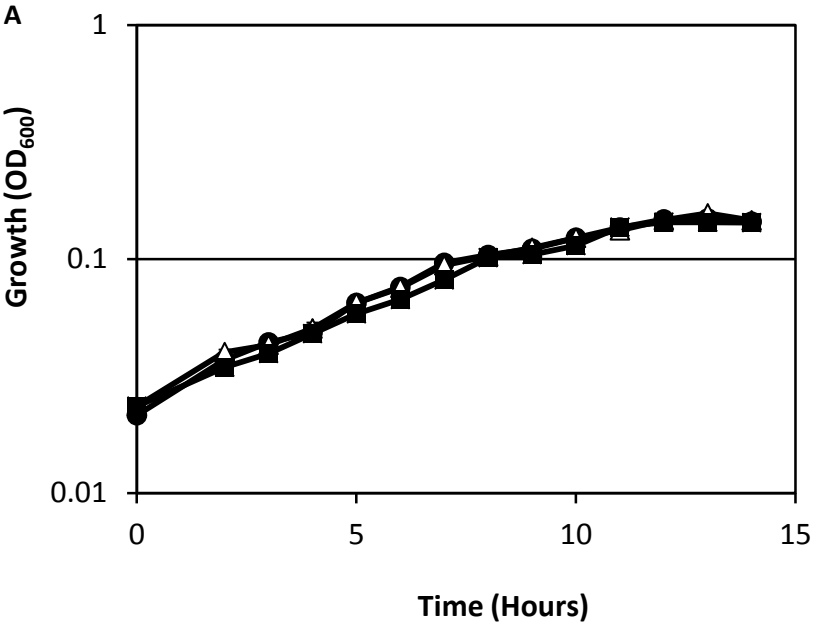
Aerobic overnight cultures of SL1344 and *ΔtolC* were grown in minimal media supplemented with metals where stated, washed with EDTA and lysed with nitric acid and intracellular metal levels quantified by ICP–MS analysis and calculated as number of atoms per cell (main axis) and number of atoms per mg of protein (secondary axis). Data is the mean of three independent repeats performed in triplicate, error bars represent standard error.

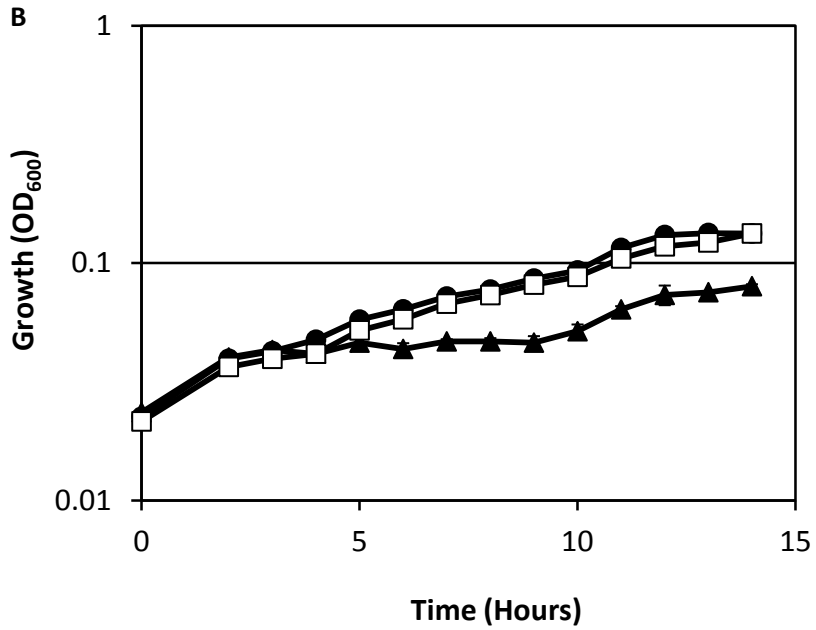
5.7 TolC does not contribute to copper tolerance under anaerobic conditions

The role of TolC contributing to copper export and tolerance under aerobic conditions has been confirmed, next the role of TolC in copper tolerance under anaerobic conditions was investigated. An aerobic overnight culture was grown in minimal media supplemented with sodium fumarate to provide an electron acceptor under anaerobic conditions. The aerobic overnight culture was used to set up an anaerobic growth curve diluting bacteria 1/50 in fresh minimal media and uptaken within a plastic syringe expelling any air bubbles and securing with an air tight Luer lock. Growth was measured hourly at OD_{600} for 15 hours until stationary phase was reached. Under anaerobic conditions *Salmonella* does not grow to the same OD_{600} value as aerobic conditions or as quickly. No difference in growth rate between SL1344 *ΔcopA/ΔgolT* and *ΔtolC* under anaerobic conditions was seen (figure 5.11A). Supplementation of 30 μM $CuSO_4$ gave decreased growth of *ΔcopA/ΔgolT* (0.08 ± 0.01) but not for SL1344 (0.13 ± 0.01) or *ΔtolC* (0.13 ± 0.01) (figure 5.11B). To confirm that the growth observed during the experiment was under anaerobic conditions a negative control of minimal media with no fumarate was performed, (figure 11C). No growth of SL1344 was seen between an OD_{600} reading at 0 hours and 28 hours confirming that the anaerobic cultures are using fumarate as a terminal electron acceptor and not oxygen. This suggests that TolC does not provide a role in copper detoxification under anaerobic conditions.

As this result was unexpected, endpoint anaerobic growth assays were also performed in rich media with SL1344 *ΔcopA/ΔgolT* and *ΔtolC*. Overnight cultures were diluted 1/100 into fresh LB and grown in varying concentrations of $CuSO_4$ until early log phase and growth was measured through absorbance OD_{600} (figure 5.12). *ΔcopA/ΔgolT* exhibited inhibited growth at 250 μM $CuSO_4$ with an OD_{600} of 0.13 (±0.02) whereas *ΔtolC* did not exhibit any difference in copper tolerance to SL1344, each having an OD_{600} of 0.21 (± 0.03) and 0.21 (±0.03) respectively. *ΔcopA/ΔgolT* had consistently lower growth when $CuSO_4$ was present in the media at all concentrations tested.

To further confirm that TolC has no role in copper tolerance under anaerobic conditions, growth assays were performed using anaerobic copper-containing LB agar plates, with SL1344 and *ΔtolC*, by serial dilution of overnight cultures to a concentration of 10^{-7}. Dilutions 10^{-7}-10^{-1} were plated onto LB agar containing varying concentrations of copper and grown overnight in an anaerobic jar under anaerobic conditions; plates were then imaged on a UV doc transilluminator (figure 5.13). No difference in growth was seen between SL1344 and *ΔtolC.* Growth curves, endpoint growth assays and monitor growth on copper-containing plates all confirm that TolC does not contribute to copper detoxification under anaerobic conditions.


A
1
0.1
0.01
Growth (OD600)
0
5
10
15
Time (Hours)


B
1
0.1
0.01
Growth (OD600)
0
5
10
15
Time (Hours)

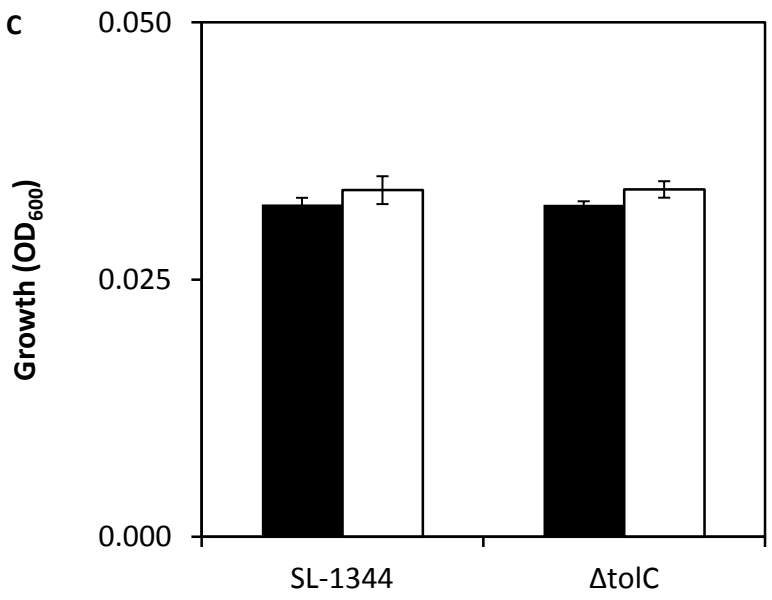


Figure 5.11 TolC does not contribute to copper tolerance of *S.* Typhimurim in minimal media under anaerobic conditions

Overnight cultures of SL1344 (●), *ΔcopA/ΔgolT* (▲) and *ΔtolC* (□) were diluted 1/100 into fresh minimal media supplemented with fumarate and incubated at 37°C statically under anaerobic conditions with, (A) no copper or (B) 30 µM $CuSO_4$. (C) Overnight cultures of SL1344 and *ΔtolC* diluted 1/100 into fresh minimal media containing no fumarate at time 0 (black) and after 15 hours (white). Growth was measured at $OD_{600.}$ Data points represent the mean of one repeat performed in triplicate, error bars represent standard error.

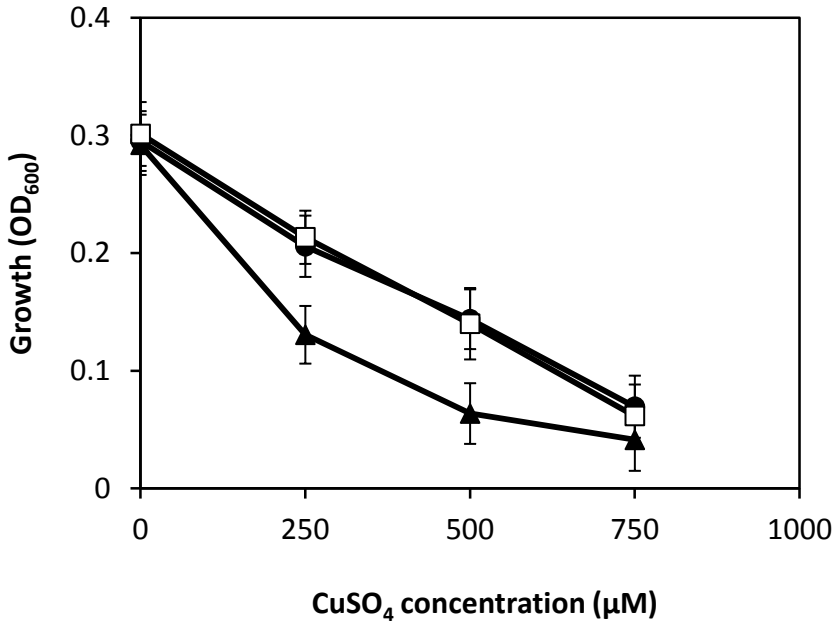


Figure 5.12 A *tolC* mutant has a similar copper tolerance to that of SL1344 in LB media under anaerobic conditions

Overnight cultures of cultures of SL1344 (●), *ΔcopA/ΔgolT* (▲) and *ΔtolC* (□) diluted 1/100 into fresh LB media with varying $CuSO_4$ concentrations and incubated at 37°C statically for 3.5 hours, growth was measured at OD_{600}. Data points represent the mean of three independent repeats performed in triplicate, error bars represent standard error.

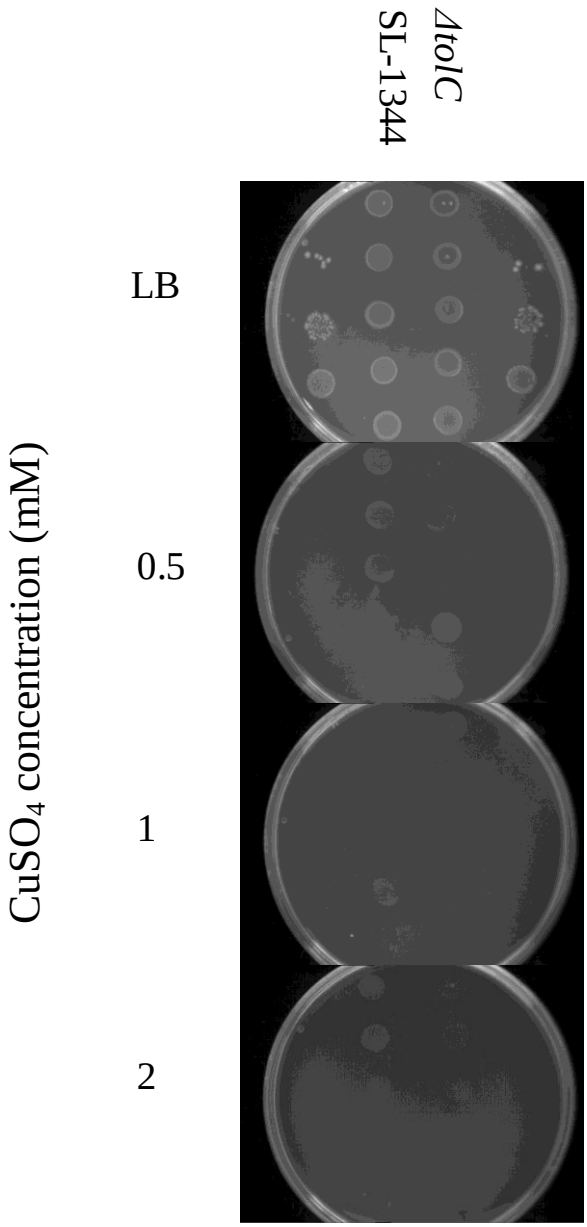


Figure 5.13 TolC does not contribute to anaerobic copper detoxification in S. Typhimurium

Overnight cultures of SL1344 and *ΔtolC* were serially diluted from 10^{0}–10^{-7} plated onto LB agar plates containing varying $CuSO_4$ concentrations. Plates were incubated statically at 37°C for 48 hours in an anaerobic jar under anaerobic conditions and imaged using a UV light transilluminator. Data is a representative of three independent repeats.

5.8 TolC does not contribute to copper export under anaerobic conditions

To assess if TolC contributes to copper export under anaerobic conditions ICP-MS analysis was performed on culture grown with and without copper supplementation (table 5.4). No difference in the copper contents was seen between SL1344 and *ΔtolC* when grown in minimal media or when grown in minimal media supplemented with 10 μM $CuSO_4$. Under anaerobic conditions *Salmonella* uptakes significantly greater amounts of copper than under aerobic growth conditions, this is consistent with observations in the literature (Outten *et al.* 2001). SL1344 grown in minimal media under aerobic growth accumulates 3.38 (± 2.74) atoms per cell compared to 7.78 (± 4.63) $x10^3$ atoms per cell under anaerobic conditions. When grown in minimal media supplemented with 10 μM $CuSO_4$, SL1344 accumulates substantially more copper under anaerobic conditions 2643.24 (± 1168.46) atoms per cell $x10^3$ than under aerobic conditions, 14.15 (± 3.56) $x10^3$ atoms per cell ($p<0.05$). The lack of difference between SL1344 and *ΔtolC* levels of copper accumulation identified that TolC does not export copper under anaerobic conditions.

The lack of export under anaerobic conditions suggests TolC only transports Cu^{2+} not Cu^{+} ions. It is believed that the periplasm reflects the oxidative state of the external environment due to the porous nature of the outer membrane. Under oxidising conditions Cu^{2+} ions are prevalent in aerobic cultures adversely under anaerobic conditions Cu^{+} ions are prevalent. TolC might not be capable of exporting Cu^{+} ions or the proteins which supply copper to TolC only supply Cu^{2+} ions.

	Atoms per cell (x10^3)		Atoms per mg protein (x10^3)	
	Cu	10 µM Cu	Cu	10 µM Cu
SL1344	7.78 (± 4.63)	2643.25 (± 1146.17)	19.09 (± 7.25)	9866.98 (± 4092.61)
ΔtolC	10.56 (± 5.54)	2781.46 (± 1168.46)	21.21 (± 8.05)	9438.06 (± 3684.29)

Table 5.4 *ΔtolC* and SL1344 have similar copper contents under anaerobic conditions

Anaerobic overnight cultures of SL1344 and *ΔtolC* grown in minimal media, supplemented with 10 µM $CuSO_4$ where stated, washed with EDTA and lysed with nitric acid. Intracellular metal levels were quantified by ICP–MS analysis and quantified by number of atoms per cell and number of atoms per mg of protein. Data is the mean of three independent repeats performed in triplicate, error bars represent standard error.

5.9 The addition of a *cueO* mutation to a *tolC* mutant gives a decrease in aerobic copper tolerance

The exportation of copper under aerobic conditions by TolC but not under anaerobic conditions, suggests specific transport of Cu^{2+}. The multicopper oxidase (CueO) is located in the periplasm and performs the oxidation of Cu^{+} to Cu^{2+} which is believed to reduce the ability of copper to re-enter the cytosol. The conversion of Cu^{+} to Cu^{2+} by CueO may be essential for the exportation of copper by TolC. To investigate whether CueO and TolC function together to achieve copper detoxification a *ΔtolC/ΔcueO* strain was made by transferring a *tolC* mutation into a *cueO* mutant by P22 phage transduction.

Endpoint copper tolerance growth assays were performed with SL1344, *ΔtolC, ΔcueO* and *ΔtolC/ΔcueO*. Overnight cultures were diluted 1/100 into fresh minimal media and grown in varying concentrations of $CuSO_4$ until early log phase, growth was quantified by measuring absorbance at OD_{600} (figure 5.14). *ΔtolC/ΔcueO* exhibited inhibited growth at 10 μM $CuSO_4$ with an OD_{600} of 0.25 (±0.04) whereas *ΔtolC* and *ΔcueO* did not exhibit any difference in growth to SL1344 OD_{600} of 0.32 (±0.01), each mutant having an OD_{600} of 0.31 (±0.03) and 0.29 (±0.02), respectively. *ΔtolC/ΔcueO* had consistently lower growth for all $CuSO_4$ concentrations examined. *ΔtolC* and *ΔcueO* both showed decreased growth in comparison to SL1344 at the higher copper concentrations used, with the largest difference seen at 75 μM $CuSO_4$. *ΔtolC* and *ΔcueO* both had similar copper tolerance to each other with *ΔcueO* having a slight reduction in growth at 10 and 25 μM $CuSO_4$ in comparison to *ΔtolC*.

To further analyse the additive nature of a *cueO* and *tolC* mutation growth on copper-containing LB agar plates for SL1344, *ΔcueO, ΔtolC, ΔtolC/ΔcueO* by serial dilution of overnight cultures to a concentration of 10^{-6}. Dilutions 10^{-6}-10^{-2} were plated onto LB agar containing varying concentrations of $CuSO_4$ and grown overnight; plates were imaged on a UV doc transilluminator (figure 5.15). *ΔtolC/ΔcueO* was highly sensitive to copper toxicity with no growth on the 2 mM $CuSO_4$ LB agar plate. Both *ΔtolC* and *ΔcueO* exhibited killing at 3 mM $CuSO_4$, *ΔtolC* only had growth at 10^{-2} dilution whereas *ΔcueO* had no visible growth and could not survive at 3 mM $CuSO_4$. SL1344 did not exhibit any killing at 4 mM $CuSO_4$ and *ΔtolC* had growth only at the 10^{-2} dilution identifying that a mutation of *cueO* reduces copper tolerance greater than mutation of *tolC*. Crucially, mutants lacking *cueO* and *tolC* are much more sensitive to copper than either single mutant.

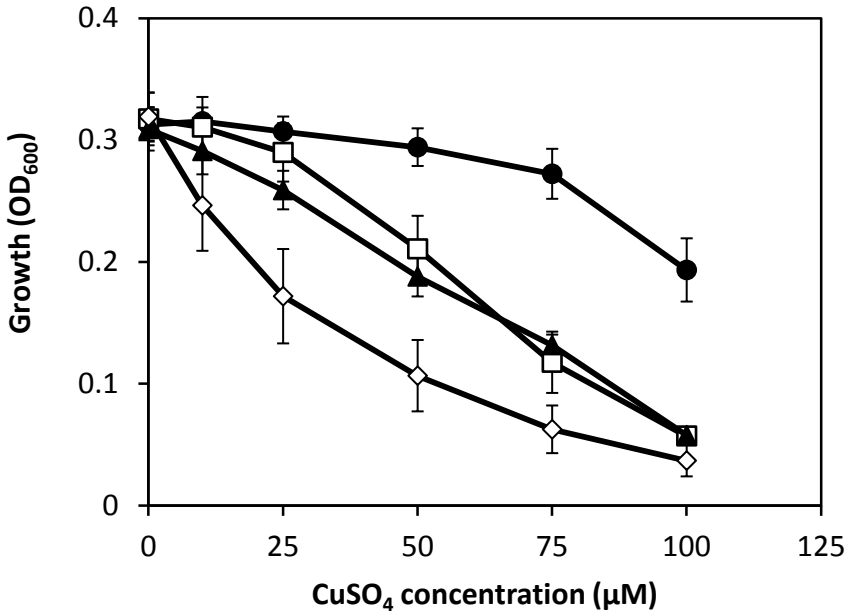


Figure 5.14 The addition of a *tolC* mutation lowers copper tolerance of *ΔcueO*

Overnight cultures of SL1344 (●), *ΔtolC* (□), *ΔcueO* (▲) and *ΔtolC/ΔcueO* (◊), diluted 1/100 into fresh minimal media with varying $CuSO_4$ concentrations and incubated at 37°C, 200 rpm for 4-5 hours under aerobic conditions, growth measured at OD_{600}. Data points represent the mean of three independent repeats performed in triplicate, error bars represent standard error.

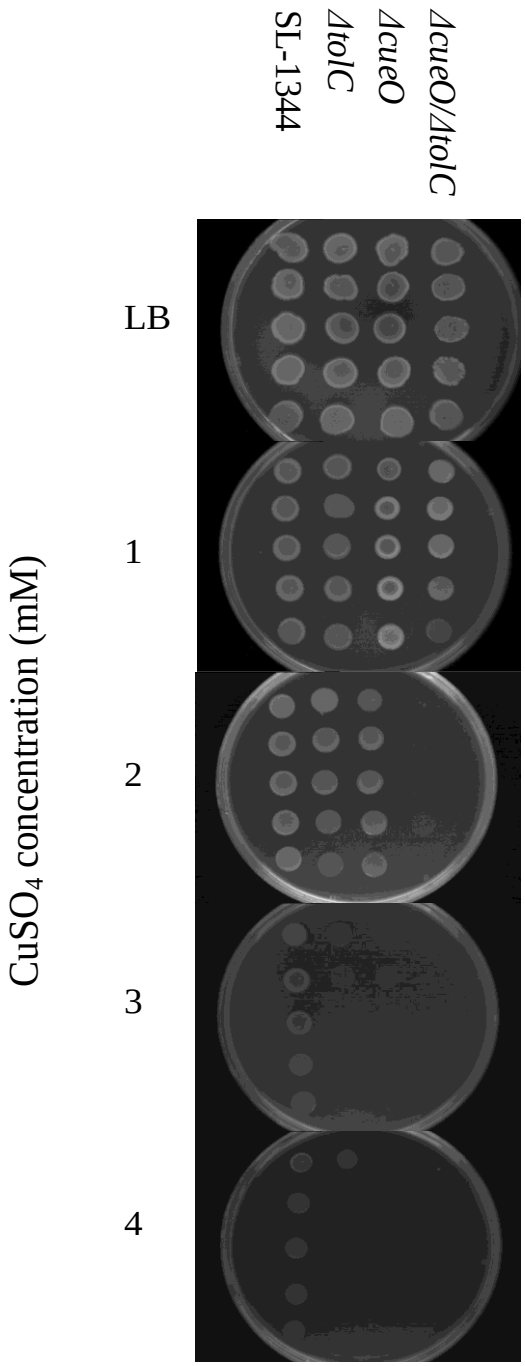


Figure 5.15 *ΔtolC/ΔcueO* is killed under low copper sulphate concentrations

Overnight cultures of SL1344, *ΔtolC, ΔcueO* and *ΔtolC/ΔcueO* were serially diluted from 10^{-2}–10^{-6} plated onto LB agar plates containing varying $CuSO_4$ concentrations. Plates were incubated statically at 37°C for 48 hours under aerobic conditions and imaged using a UV light transilluminator. Data is a representative of three independent repeats.

The increased copper sensitivity of a *tolC/cueO* double mutant compared to single mutants indicates that TolC is not dependent on CueO to function but both are important periplasmic copper detoxifying components. The copper-containing LB agar plate assay also confirmed that a *tolC/cueO* double mutant had reduced copper tolerance than a *copA/golT* double mutant; which grew at a 10^{-4} dilution when exposed to 2 mM $CuSO_4$ whereas *ΔtolC/ΔcueO* was killed. This highlights that copper has a harmful affect on *Salmonella* from outside the cytosol, where current toxicity models for copper include: Fenton chemistry, iron sulphur cluster disruption and metalloprotein disruption. CueO detoxifies Cu^{+} to Cu^{2+} in the periplasm and it is possible that TolC exports Cu^{2+} that CueO has converted but TolC is not solely reliant on receiving Cu^{2+} from CueO as indicated by the additive nature of a *tolC/cueO* double mutant. It is likely that TolC is able to bind Cu^{2+} that is present in the periplasm from either the oxidation of Cu^{+} in the periplasm by CueO or alternate proteins with oxidising capabilities or Cu^{2+} that has entered the periplasm from the external environment through the outer membrane.

5.10 CueP and TolC do not function together to provide copper tolerance or homeostasis

5.10.1 CueP is not required for TolC mediated copper resistance

As previously stated; genes encoding RND efflux systems that require TolC as an outer membrane exporter cluster across several Gram negative bacteria close to a gene encoding of similar sequence to CueP. The *cus* system within *E. coli* requires the presence of CusF, a periplasmic copper chaperone that transports Cu^{+} to CusB for export by CusC. *Salmonella* does not possess the Cus system although CueP bears a similarity to CusF in that it is a periplasmic copper binding protein that has a known copper chaperone function (Osman *et al.* 2013, Bagai *et al.* 2008). TolC may receive copper from the cytosol by the switch mechanism or from the periplasm through the funnel mechanism. The funnel mechanism may therefore be dependent on a periplasmic copper chaperone transporting copper to TolC that CueP could potentially perform. A *tolC* mutation was transferred into a *cueP* mutant by P22 phage transduction and the resulting *ΔtolC/ΔcueP* mutant copper tolerance was analysed under both aerobic and anaerobic conditions.

Endpoint copper tolerance growth assays were performed with SL1344, *ΔtolC, ΔcueP* and *ΔtolC/ΔcueP*. Overnight cultures were diluted 1/100 into fresh minimal media and grown in varying concentrations of $CuSO_4$ until early log phase and growth was measured by absorbance at OD_{600} (figure 5.16). As stated in previous work (Osman *et al.* 2010) *ΔcueP* did

not show any difference in copper tolerance to SL1344 giving an OD_{600} of 0.184 (± 0.01) and 0.183 (± 0.01) respectively. *ΔtolC/ΔcueP* exhibited no difference in copper tolerance to *ΔtolC* with an OD_{600} value of 0.104 (± 0.01) and 0.103 (± 0.01) respectively at 100 μM $CuSO_4$. To test whether or not CueP contributes to copper tolerance in the presence of rich media under aerobic conditions, copper-containing LB agar plate assays were performed. Overnight cultures of SL1344, *ΔcueP, ΔcueP/ΔcueO* and *ΔtolC/ΔcueP* were diluted by serial dilution to a concentration of 10^{-6}. Dilutions 10^{-6}-10^{-2} were plated onto LB agar containing varying concentrations of copper and grown overnight; plates were imaged on a UV doc transilluminator (figure 5.17). As seen with minimal media endpoint growth assays no difference was seen between SL1344 and *ΔcueP*. Regarding copper tolerance no difference in killing was present between *ΔcueP/ΔcueO* and *ΔcueO* or *ΔtolC/ΔcueP* and *ΔtolC* (figures 5.14 and 5.15). This confirmed under aerobic conditions CueP does not provide copper tolerance to S. Typhimurium or influence the copper tolerance conferred by CueO or TolC.

5.10.2 CueP does not contribute to copper export under non-toxic copper concentrations

After confirming CueP does not provide a role in copper tolerance under aerobic conditions, it was investigated to identify if CueP performs a role in copper export under these conditions. ICP-MS analysis was performed with SL1344, *ΔtolC, ΔcueP* and *ΔtolC/ΔcueP* grown in minimal media supplemented with 10 μM $CuSO_4$ (table 5.5). No difference was seen in the copper contents between SL1344 and *ΔcueP* when the data was expressed as atoms per cell or atoms per mg protein. Also no difference was seen between *ΔtolC* and *ΔtolC/ΔcueP*, both strains accumulated similar levels of copper, greater than SL1344, due to the mutation of *tolC*. Through the combination of the minimal media endpoint growth assay, rich media solid agar plates and ICP-MS analysis it can be concluded that CueP does not influence copper homeostasis or tolerance under aerobic conditions.

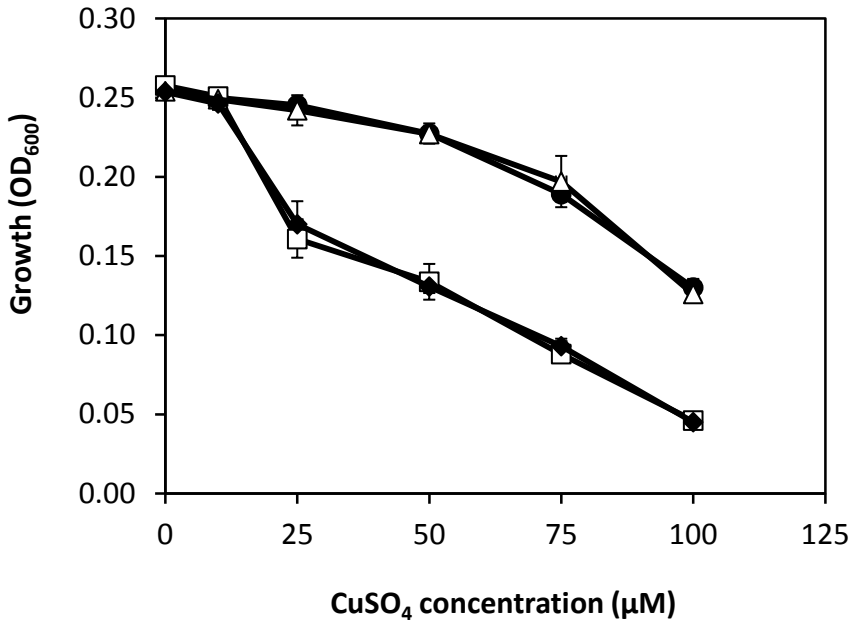


Figure 5.16 CueP does not provide copper tolerance to S. Typhimurium under aerobic conditions

Overnight cultures of SL1344 (●), *ΔtolC* (□), *ΔcueP* (Δ) and *ΔtolC/ΔcueP* (♦) diluted 1/100 into fresh minimal media with varying $CuSO_4$ concentrations and grown at 37°C 200 rpm for 4-5 hours, growth was measured at OD_{600}. Data points represent the mean of one repeat performed in triplicate, error bars represent standard error.

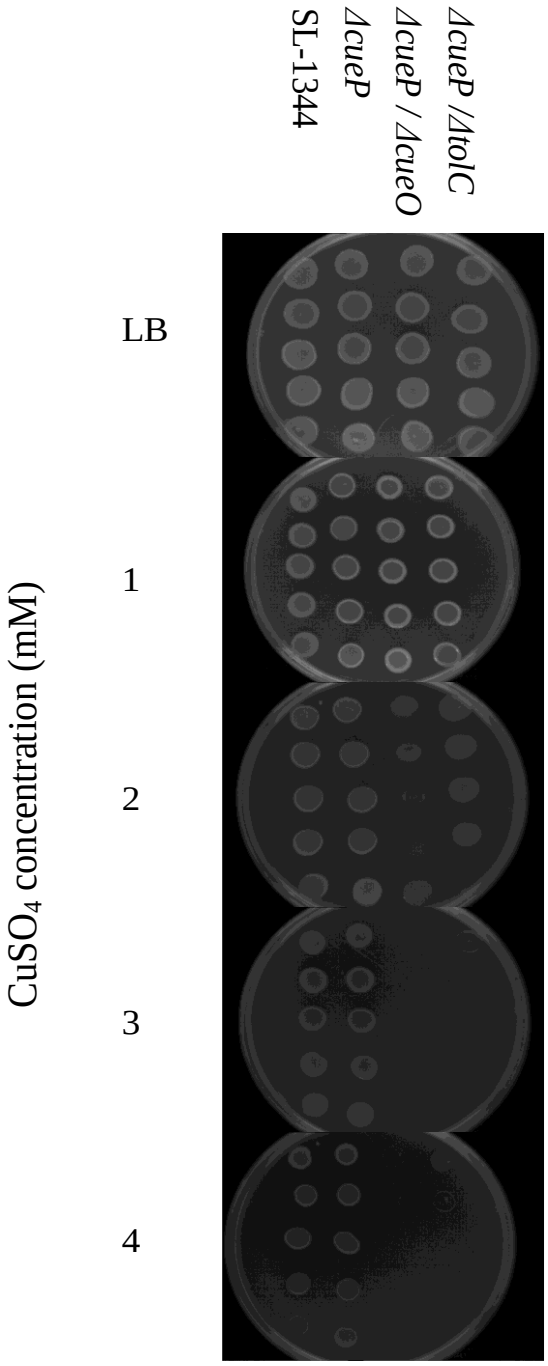


Figure 5.17 CueP does not provide a role in aerobic copper tolerance or influence CueO or TolC

Overnight cultures of SL1344, *ΔcueP, ΔcueP/ΔcueO* and *ΔcueP/ΔtolC* were serially diluted from 10^{-2}–10^{-6} plated onto LB agar plates containing varying $CuSO_4$ concentrations. Plates were incubated statically at 37°C for 48 hours and imaged using a UV light transilluminator. Data is a representative of three independent repeats.

	Atoms per cell (x10^3)				
	Mn	Co	Ni	Cu	Zn
SL1344	1.81 (± 0.01)	0.00 (± 0.00)*	1.8 (± 0.02)	16.09 (± 0.42)	7.28 (± 0.20)
ΔtolC	1.97 (± 0.36)	0.00 (± 0.00)*	1.68 (± 0.03)	68.06 (± 0.71)	11.47 (± 2.01)
ΔcueP	1.67 (± 0.17)	0.00 (± 0.00)*	2.66 (± 0.63)	14.48 (± 1.24)	12.49 (± 3.70)
ΔtolC/ΔcueP	1.85 (± 0.52)	0.00 (± 0.00)*	2.27 (± 0.14)	66.8 (± 0.88)	19.14 (± 3.20)

	Atoms per mg protein (x10^3)				
	Mn	Co	Ni	Cu	Zn
SL1344	7.4 (± 0.07)	0.00 (± 0.00)*	7.4 (± 0.11)	66.03 (± 2.96)	29.85 (± 1.45)
ΔtolC	7.13 (± 2.21)	0.00 (± 0.00)*	6.06 (± 0.19)	246.23 (± 4.30)	41.5 (± 12.20)
ΔcueP	5.84 (± 1.08)	0.00 (± 0.00)*	9.31 (± 3.95)	50.78 (± 7.76)	43.78 (± 23.18)
ΔtolC/ΔcueP	7.54 (± 3.66)	0.00 (± 0.00)*	9.26 (± 0.98)	272.3 (± 6.21)	78.02 (± 22.58)

Table 5.5 CueP does not contribute to copper homeostasis under non-toxic copper levels

Aerobic overnight cultures of SL1344, *ΔtolC, ΔcueP* and *ΔtolC/ΔcueP* were grown in minimal media supplemented with 10 µM copper, washed with EDTA and lysed with nitric acid. Intracellular metal levels quantified by ICP–MS analysis and calculated as number of atoms per cell (main axis) and number of atoms per mg of protein (secondary axis). Data is the mean of three independent repeats performed in triplicate, error bars represent standard error. *Cobalt below the detectable limit

5.10.3 A *cueP* mutant has similar copper tolerance to SL1344 under anaerobic conditions

It has been reported (Pontel and Soncini 2009) that CueP contributes to copper tolerance under anaerobic conditions, this result and whether CueP is able to provide copper tolerance in a *tolC* mutant was investigated. Endpoint copper tolerance growth assays were performed under anaerobic conditions with SL1344, *ΔtolC, ΔcopA/ΔgolT*, *ΔcueP* and *ΔtolC/ΔcueP*. Overnight cultures were diluted 1/100 into fresh LB and grown in varying concentrations of $CuSO_4$ until early log phase and growth was measured through absorbance at OD_{600} (figure 5.18). *ΔcopA/ΔgolT* exhibited inhibited growth at 250 μM $CuSO_4$ with an OD_{600} of 0.16 (± 0.03) $CuSO_4$ whereas *ΔcueP* and *ΔtolC* did not exhibit any difference in copper tolerance to SL1344 each having an OD_{600} of 0.22 (± 0.02) and 0.24 (± 0.03), respectively and SL1344 0.23 (± 0.03). This suggests that CueP does not have a role in copper tolerance under anaerobic conditions. To further analyse the role of CueP under anaerobic conditions, growth curves were also performed in minimal media supplemented with copper.

An aerobic overnight culture was grown in minimal media supplemented with sodium fumarate and was used to set up an anaerobic growth curve diluting bacteria 1/50 into fresh minimal media (figure 5.19A) and minimal media supplemented with 30 μM $CuSO_4$ (figure 5.19B) and uptaken within a plastic syringe expelling any air bubbles and securing with an air tight Luer lock. Growth was measured at OD_{600} for 14 hours until stationary phase was reached. No difference in growth rate between SL1344 *ΔcopA/ΔgolT*, *ΔtolC, ΔcueP* and *ΔtolC/ΔcueP* was seen in minimal media without added copper. Supplementation of 30 μM $CuSO_4$ into minimal media gave decreased growth of *ΔcopA/ΔgolT*, but not for SL1344, *ΔtolC, ΔcueP* and *ΔtolC/ΔcueP*. To confirm that the growth observed during the experiment was under anaerobic conditions, a negative control of minimal media with no fumarate was performed (figure 19C). No growth of SL1344 was seen between an OD_{600} reading at 0 hours and 28 hours confirming that the anaerobic cultures are using fumarate as a terminal electron acceptor and not oxygen. This supports the assertion that TolC and CueP do not provide a role in copper detoxification under anaerobic conditions.

It can be concluded CueP does not provide a role in copper homeostasis or copper tolerance under anaerobic conditions. Recently CueP has been identified to transport copper to $SodC_{II}$, it is most likely the copper binding capacity of CueP is associated with its role as a copper chaperone and not part of the primary copper detoxification response in S. Typhimurium (Osman *et al.* 2013).

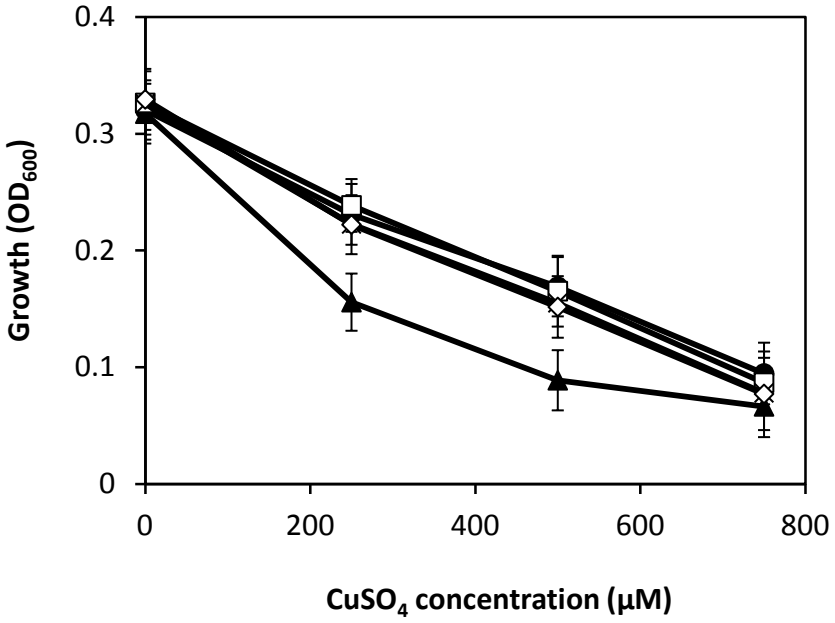
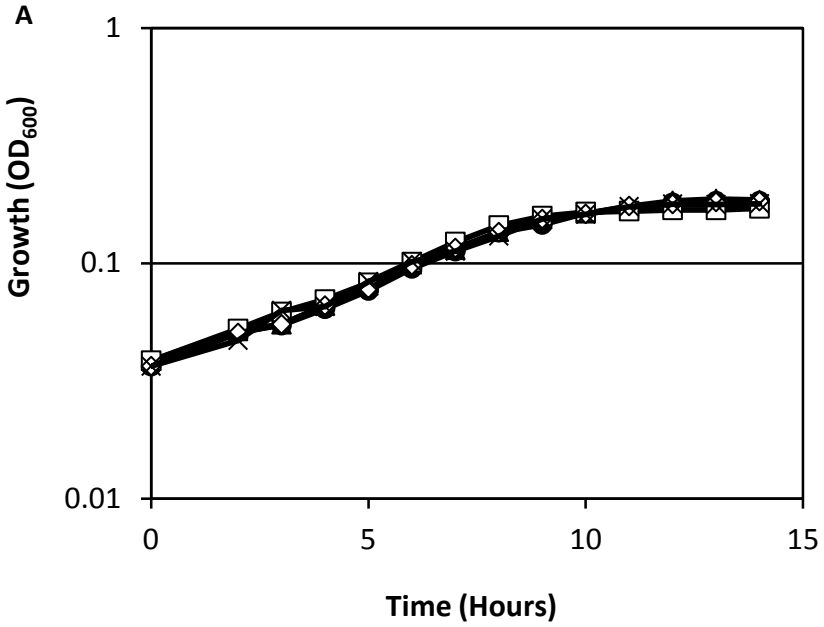
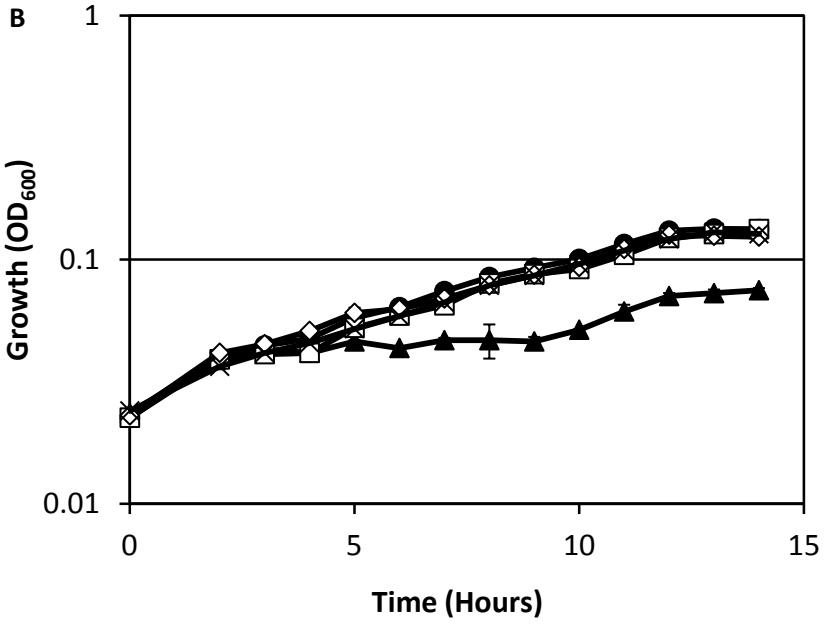


Figure 5.18 A *cueP* mutation does not provide copper tolerance under anaerobic conditions

Overnight cultures of SL1344 (●), *ΔtolC* (□), *ΔcopA/ΔgolT* (▲), *ΔcueP* (**X**) and *ΔcueP/ΔtolC* (◊) diluted 1/100 into fresh LB media with varying $CuSO_4$ concentrations and incubated statically at 37°C for 3.5 hours, growth was measured at OD_{600}. Data points represent the mean of three independent repeats performed in triplicate, error bars represent standard error.


A
1
0.1
0.01
Growth (OD600)
0
5
10
15
Time (Hours)


B
1
0.1
0.01
Growth (OD600)
0
5
10
15
Time (Hours)

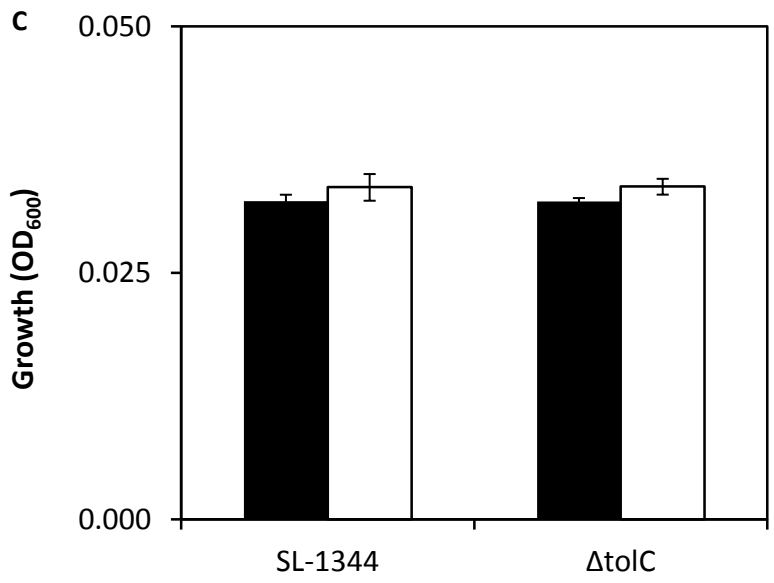


Figure 5.19 TolC and CueP do not contribute copper tolerance under anaerobic conditions in S. Typhimurium

Overnight cultures of SL1344 (●), *ΔcopA/ΔgolT* (▲), *ΔtolC* (□), *ΔcueP* (◊) and *ΔtolC/ΔcueP* (**X**) were diluted 1/100 into (A) fresh minimal media containing sodium fumarate and grown at 37°C statically or (B) supplemented with 30 µM copper sulphate. (C) Overnight cultures of SL1344 and *ΔtolC* diluted 1/100 into fresh minimal media containing no fumarate at time 0 (black) and after 28 hours (white). Growth was measured at OD_{600}. Data points represent the mean one repeat performed in triplicate, error bars represent standard error.

5.11 The Ges system does not contribute to copper tolerance within S. Typhimurium

Another RND efflux system within *Salmonella* is the *ges* system that consists of *gesABC*; GesA is an inner membrane transporter, GesB a membrane fusion protein and GesC an outer membrane transporter. The *ges* system has been identified to be involved in the export of gold and numerous antibiotics (Pontel *et al.* 2007). TolC is capable of functionally replacing GesC which has led into the investigation that Ges system could potentially export copper (Pontel *et al.* 2007). Therefore, GesC could functionally substitute for TolC in a *tolC* mutant with respect to copper export, which is not associated with Ges A or Ges B. Aerobic and anaerobic endpoint assays were performed to analyse if the Ges system can export copper.

A *gesB/gesC* double mutant was generated by insertional mutagenesis as described in Datsenko and Wanner (2000). Aerobic endpoint copper tolerance growth assays were performed with SL1344, *ΔtolC, ΔgesB/ΔgesC*. Overnight cultures were diluted 1/100 into fresh minimal media and grown in varying concentrations of $CuSO_4$ until early log phase and growth was measured by absorbance at OD_{600} (figure 5.20A). Anaerobic endpoint copper tolerance growth assays were performed under anaerobic conditions with SL1344, *ΔtolC, ΔgesB/ΔgesC*. Overnight cultures were diluted 1/100 into fresh LB and grown in varying concentrations of $CuSO_4$ until early log phase and growth was measured through absorbance at OD_{600} (figure 5.20B).

No difference was seen between a GesBC mutant and SL1344 under aerobic or anaerobic conditions. This indicated that the Ges system is unable to export copper and TolC does not provide copper tolerance through the supply of copper from GesB. During the study a triple mutant of *ΔgesB/ΔgesC/ΔtolC* was attempted but was unable to be created, it is possible that by removing both outer membrane exporters GesC and TolC reduces membrane stability in S. Typhimurium. A deletion of *tolC* and *gesC* would identify if GesC can associate with an alternate cytosolic transport and membrane fusion protein and export copper, if a decrease in copper tolerance was exhibited.

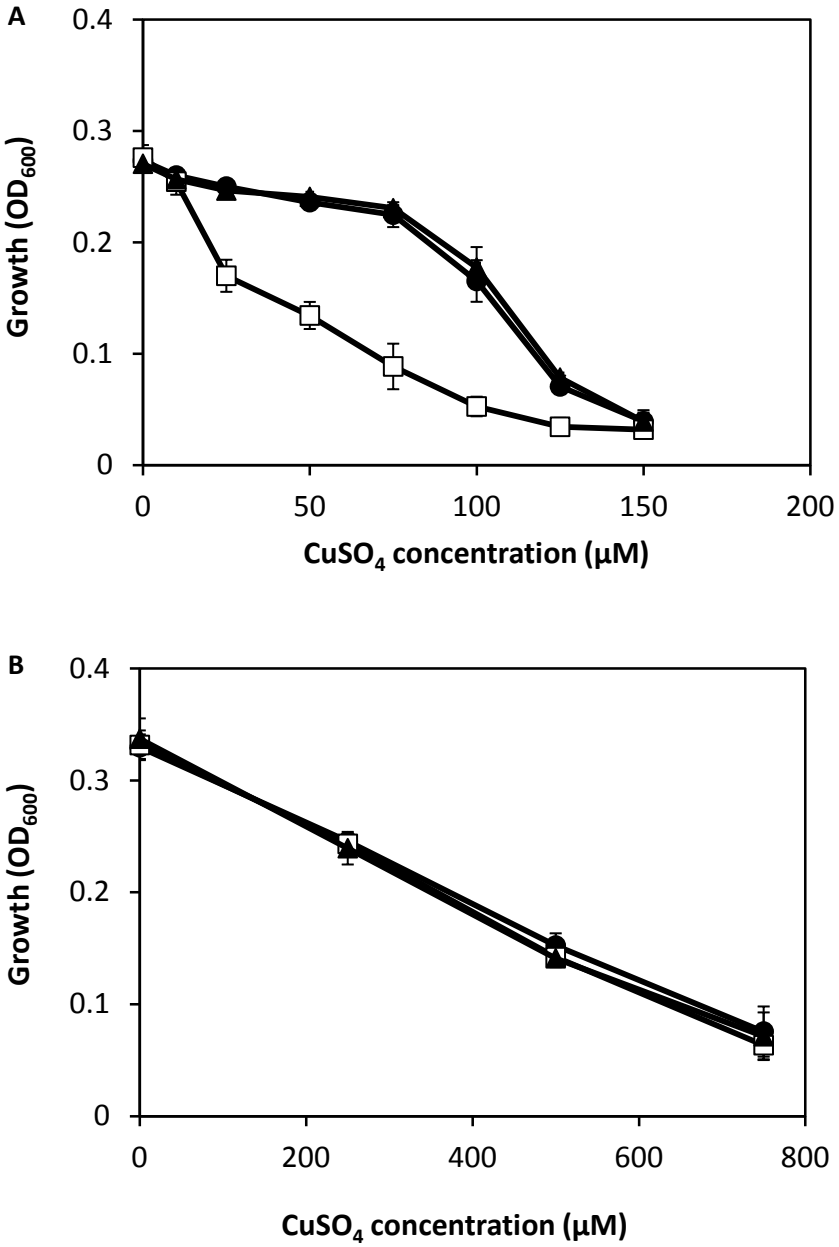


Figure 5.20 GesBC does not contribute to copper homeostasis in S. Typhimurium under both aerobic and anaerobic conditions

Overnight cultures of SL1344 (●), *ΔtolC* (□) and *ΔgesBC* (▲), (A) diluted 1/100 into fresh minimal media with varying $CuSO_4$ concentrations and grown at 37°C 200 rpm for 4-5 hours. (B) Diluted 1/100 into fresh LB with varying copper concentrations and incubated at 37° for 3.5 hours. Growth measured at OD_{600}. Data points represent the mean of three independent repeats performed in triplicate, error bars represent standard error.

5.12 Discussion

5.12.1 TolC provides a role in S. Typhimurium copper homeostasis when grown under aerobic conditions but not when grown under anaerobic conditions

The data obtained in this study indicates that TolC is required for copper homeostasis under aerobic conditions. A *tolC* mutant was shown to have decreased growth compared to SL1344 in the presence of copper. When assayed by endpoint growth assays in minimal media, *ΔtolC* grew similar to SL1344 until a concentration of 25 µM $CuSO_4$ was reached, then a substantial reduction in growth occurred. (figure 5.4). During growth curve analysis *ΔtolC* also grew at a slower rate than SL1344 in the presence of 50 µM $CuSO_4$ in growth curve experiments (figure 5.7). Growth on copper-containing LB agar plates (figure 5.5) at 3 mM and 4 mM $CuSO_4$ exhibited a four log difference was present between SL1344 and *tolC* confirming previous data in minimal media for endpoint and growth curve experiments that *ΔtolC* has reduced copper tolerance than SL1344 under aerobic conditions.

Complementation of *tolC* into a *tolC* mutant restored copper tolerance similar to that of SL1344 during endpoint and copper containing LB agar plates assays (figure 5.4 and 5.5), confirming that TolC is providing copper tolerance under aerobic conditions within S. Typhimurium. A *tolC* mutant was previously reported to have reduced growth in the presence of copper and zinc under aerobic conditions (Nishino *et al.* 2007). The data obtained in this study is consistent with the reported copper phenotype identified in a *tolC* mutant. After confirming the importance of TolC in copper tolerance its importance in copper homeostasis was investigated by ICP-MS analysis of cellular copper contents. The addition of 10 µM $CuSO_4$ to SL1344 and *ΔtolC* grown overnight in minimal media identified a *tolC* mutant over accumulated 4.1 fold increase (atoms per cell) copper in comparison to SL1344 (figure 5.9). This identified that TolC provides copper export to reduce cellular copper load before toxic copper levels are reached by S. Typhimurium (<10 µM $CuSO_4$).

After establishing the role of TolC in copper tolerance and export under aerobic conditions, the function of TolC was investigated under anaerobic conditions. As performed under aerobic conditions endpoint growth assays, growth curves and growth on LB agar plates containing copper were performed. No difference between SL1344 and *ΔtolC* in: copper tolerance for endpoint growth assays in minimal media (figure 5.11), in duration taken to reach stationary phase during growth curves (figure 5.12) and growth on LB agar plates containing copper (figure 5.13).To confirm copper-mediated toxicity under these conditions a *copA/golT* double mutant was utilised as a positive control and had reduced growth in

endpoint assays (figure 5.11) and slower growth rate in growth curves (figure 5.12). The data obtained identified TolC does not perform a role in copper tolerance under anaerobic conditions. To further analyse the role of TolC under anaerobic conditions copper export was investigated by ICP-MS analysis of cellular copper contents. No difference was seen between the copper quota of SL1344 and *ΔtolC* under anaerobic conditions (table 5.3). Both SL1344 and *ΔtolC* accumulated significantly greater concentrations of copper under anaerobic conditions. This confirmed data seen by other research groups that under anaerobic conditions intracellular copper levels increase in comparison to aerobic intracellular copper levels (Outten *et al.* 2001). It has been suggested that Cu^{+} has increased ability to enter the cytosol than Cu^{2+} due Cu^{+} having a smaller charge and therefore can pass through the lipohilic cytoplasmic membrane with greater ease then the more polar Cu^{2+} ion (Outten *et al.* 2001). ICP-MS data confirmed anaerobic growth data that TolC does not provide a role in copper detoxification under anaerobic conditions. It is possible that another outer membrane exporter is upregulated under anaerobic conditions that exports copper.

TolC does not directly select substrate which it exports, selection is controlled by either a cytoplasmic transporter or a membrane fusion protein that associate with TolC depending on the mechanism of export. If a cytoplasmic transporter is providing selection the substrate is transported directly from the cytosol to the external environment (switch mechanism). If the membrane fusion protein provides selection it binds the substrate to be transported within the periplasm and associates with TolC which then exports the substrate (funnel mechanism, Kim *et al.* 2011). It is likely that the selective component of the TolC copper efflux system does not bind copper under anaerobic conditions or more specifically Cu^{+}. Under anaerobic conditions Cu^{+} is prevalent, whereas under aerobic conditions Cu^{2+} is present. Therefore based on the data obtained it can be inferred that TolC is a Cu^{2+} specific transporter. Experimental evidence for a Cu^{2+} specific exporter has not previously been reported, although the existence of potential Cu^{2+} exporters have been suggested (Whittal *et al.* 2000). Cu^{+} transporters have been identified in several Gram negative bacteria including the exporters: CopA, CopB, GolT, CusA and CusC found within *E. coli*, S. Typhimurium and *Enterococcus hirae* (Outten *et al.* 2001, Osman *et al.* 2013, Solioz and Odermatt 1994). The cytosol maintains a reduced environment through the buffering action of glutathione and thredoxin. Copper present within the cytosol is reduced to Cu^{+}; consequently copper exporting ATPases within the cytoplasmic membrane are Cu^{+} specific. The Cu^{+} specific nature of CusC is potentially determined by the cytoplasmic transporter CusA that exports copper from the cytosol with CusC exporting Cu^{+} across the periplasmic membrane. Also CusF binds Cu^{+} in the periplasm and may directly receive Cu^{+} from a cytoplasmic exporter to prevent oxidation of Cu^{+} to Cu^{2+}. The model of the Cus system suggests that the TolC efflux

system present in *S. typhimurim* does not obtain Cu^+ from the cytosol and that it follows a funnel mechanism, obtaining copper from within the periplasm.

TolC was also investigated for its capability to export zinc, nickel and cobalt cations. Endpoint tolerance assays revealed no difference in tolerance between SL1344 and *ΔtolC* (figure 5.8). To further analyse the ability of TolC to export zinc, nickel and cobalt, ICP-MS analysis of cellular metal contents was performed using cultures grown at sub-lethal concentrations of these cations (table 5.2). ICP-MS data showed no difference in accumulation of zinc, nickel and cobalt between SL1344 and *ΔtolC*. Previous work identified a S. Typhimurium *ΔtolC* strain to have reduced tolerance to copper and zinc (Nishino *et al.* 2007). No difference in tolerance or accumulation of zinc by a *tolC* mutant was seen in this study to SL1344. A *tolC* mutant only exhibited both reduced tolerance and increased accumulation of copper not zinc, nickel or cobalt. Nishino *et al.* (2007) used a different strain of *S. typhimuirum*, 14028s, which could explain the difference in zinc tolerance.

5.12.2 TolC does not provide copper tolerance or homeostasis by interacting with CopA or GolT

This study has identified TolC provides copper export under aerobic conditions. It is known that TolC is required to associate with a cytoplasmic transporter and membrane fusion protein to export a substrate. The creation of a *ΔcopA/ΔgolT/ΔtolC* strain identified that a *tolC* mutation gives a reduction in copper tolerance to that of *ΔcopA/ΔgolT*. Previously it had been shown that *ΔcopA/ΔgolT* has extreme sensitivity to copper (Osman *et al.* 2010). The addition of a *tolC* mutation to *ΔcopA/ΔgolT* resulted in a strain hypersensitive to copper exhibiting a 2 log decrease in survival in comparison to a *ΔcopA/ΔgolT* strain when grown on copper-containing agar plates (figure 5.8). Liquid tolerance assays both as endpoint and growth curves also confirmed a significant reduction in growth of a *ΔcopA/ΔgolT/ΔtolC* compared to *ΔcopA/ΔgolT* in the presence of copper (figures 5.6 and 5.7). Copper accumulation was investigated to analyse if the addition of a *tolC* mutation to *copA/golT* double mutant affected copper export for cultures grown at non-toxic copper levels. ICP-MS analysis of cellular copper contents was performed with SL1344, *ΔtolC, ΔcopA/ΔgolT* and *ΔcopA/ΔgolT/ΔtolC*, it is noted that these cultures were grown at a lower concentration of copper (0.25 µM) than when *ΔtolC* copper contents were analysed (figure 5.9, 10 µM). A 10 µM $CuSO_4$ concentration would give reduced growth of *ΔcopA/ΔgolT/ΔtolC* and *ΔcopA/ΔgolT* (figure 5.6) consequently a non-toxic concentration of 0.25 µM $CuSO_4$ was

used. A slight increase in copper accumulation was seen between *ΔcopA/ΔgolT/ΔtolC* and *ΔcopA/ΔgolT* but there was no significant difference between the strains (p=0.17). The lack of significant difference for copper quota between *ΔcopA/ΔgolT/ΔtolC* and *ΔcopA/ΔgolT* could be due to TolC not contributing to copper export at a low concentration of copper as 0.25 µM, and is only required at a higher copper concentration such as 10 µM. This data indicates that CopA and GolT provide copper detoxification at a lower level of copper stress than TolC (<0.25 µM). This confirms previous work that shows copper is tightly regulated within the cytosol due to its ability when uncomplexed to disrupt iron sulphur clusters and catalyse the formation of hydroxyl radicals (Changela *et al.* 2003, Macomber an Imlay 2009). TolC associated copper tolerance is observed between 0.25-10 µM $CuSO_4$. Copper cannot be tightly regulated within the periplasm due to its porous nature. Small open channels, termed porins, are present within the outer membrane and enable diffusion of small molecules less than 600 Da in size including ions and solvents. It is believed that metals enter *Salmonella* down a concentration gradient though porins within the outer membrane (Nikado 1994). The cytoplasmic membrane contains only regulated channels providing greater control of what enters the cytosol. The periplasm is expected to have a greater copper concentration than the cytosol due to passive diffusion of copper from a copper rich environment through porins into the periplasm. Outten *et al.* (2001) suggested that the *cue* system is the principal copper detoxification system under aerobic conditions and only when the *cue* system is saturated is the *cus* system upregulated providing detoxification of the periplasm. Similarly, *Salmonella* upregulation of TolC to export copper from the periplasm may occur upon toxic levels of copper within the cytosol. The non-activity of TolC at low levels of copper may encourage retaining copper required for cuproproteins by *Salmonella*.

The additive nature of a *tolC* mutation to a *copA/golT* double mutant indicates that these copper homeostatic proteins do not form an efflux system consisting of TolC as the outer membrane exporter and either CopA or GolT as a cytoplasmic pump. It is possible for CopA/GolT and TolC to indirectly function together for copper detoxification of S. Typhimurium but non-specifically. Possible interaction of CopA, GolT and TolC are shown in (figure 5.21). The periplasm mirrors the environmental conditions in the external environment. A potential source of Cu^{2+} for TolC to export include Cu^{+} exported into the periplasm that is oxidised to Cu^{2+} under aerobic conditions by the actions of the multicopper oxidase CueO. It is unlikely CopA or GolT directly supply TolC based on data obtained in this study and that CopA/GolT are thought to export Cu^{+}.

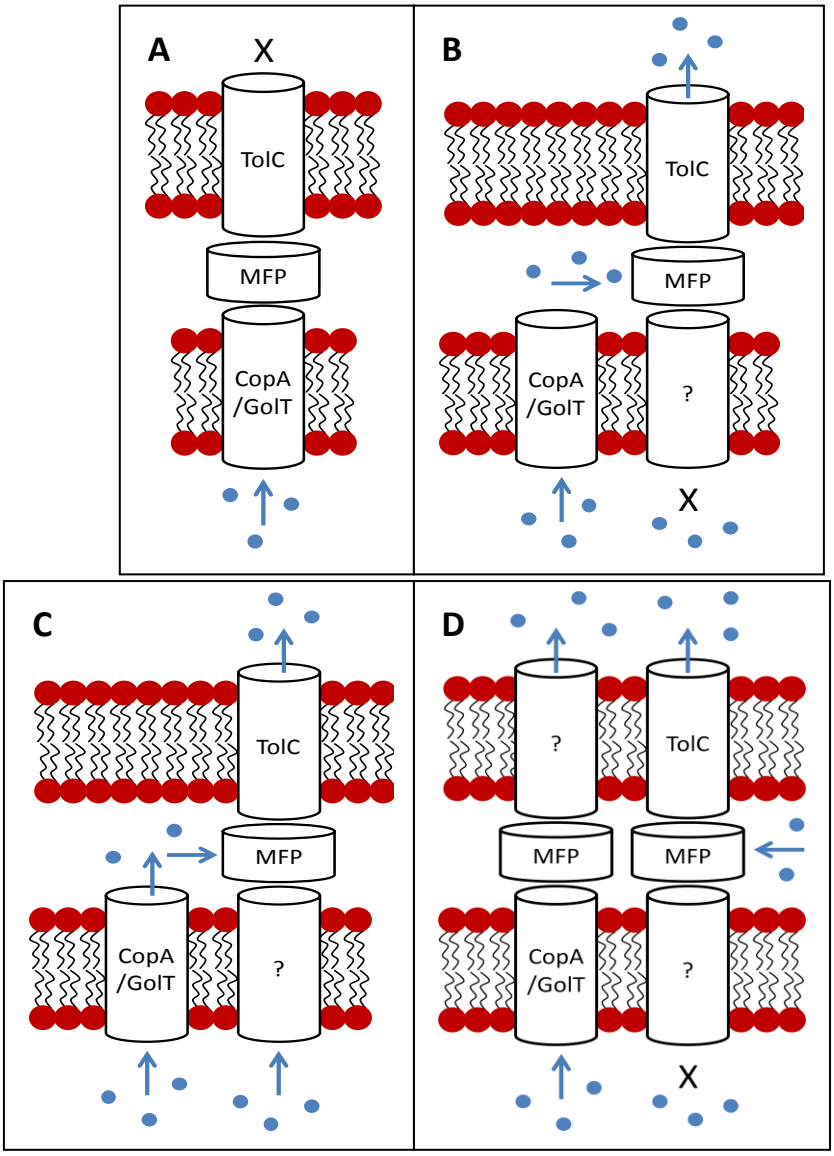


Figure 5.21 Possible alignments and interactions of TolC and CopA or GolT in S. Typhimurium

The additive nature of a *tolC* mutation to a *copA/golT* double mutation indicates that TolC does not exclusively function with CopA or GolT eliminating that they combine to form an efflux system exporting copper (A). CopA or GolT could export copper into the periplasm where a membrane fusion protein could bind copper, recruit a cytoplasmic translocase and in turn recruit TolC to enable export of copper (B). CopA and GolT provide a key role in copper export but are not the sole suppliers of copper to the membrane fusion protein. TolC may directly receive copper pumped out of the cytosol by another exporter, or receive copper bound to a membrane fusion protein that obtained copper from the actions of CopA/GolT (C). TolC and CopA/GolT may be part of independent copper detoxification systems, TolC could associate with an alternate cytoplasmic transporter that does not export copper and TolC acquires copper from the periplasm; and CopA/GolT may associate with an alternate outer membrane factor to remove copper from the periplasm (D).

5.12.3 TolC does not require CueO to provide copper tolerance under aerobic conditions

Data obtained during this study identified that TolC provides copper tolerance under aerobic conditions and not anaerobic conditions. TolC may be a Cu^{2+} transporter that is unable to transport Cu^{+}. The multicopper oxidase CueO converts Cu^{+} into Cu^{2+} within the periplasm which is thought to reduce the ability of copper to re-enter the cytosol (Outten *et al.* 2001, Grass and Rensing 2001). Cu^{2+} has increased polarity and therefore reduced ability to cross the lipophilic cytoplasmic membrane than the less charged Cu^{+}. Both *ΔtolC* and *ΔcueO* had similar copper tolerance both exhibiting increased killing in comparison to SL1344 under aerobic conditions for endpoint growth assays and copper-containing LB agar plate assays (figures 5.14 and 5.15). *ΔtolC/ΔcueO* had increased copper sensitivity than either *ΔtolC* or *ΔcueO.* The additive nature of TolC and CueO copper detoxification mechanism indicates that TolC is not reliant upon CueO to convert Cu^{+} into Cu^{2+} within the periplasm. As previously stated, copper in aerobic cultures will be in a Cu^{2+} oxidation state; Cu^{2+} that enters the periplasm from the external environment and can be removed by TolC. The reduced copper tolerance of a *cueO* mutant highlights the importance of a multicopper oxidase oxidising Cu^{+} into Cu^{2+} with respect to copper detoxification. In the absence of CueO TolC still receives Cu^{2+} but in the presence of CueO it is likely a greater proportion of the periplasmic copper content is Cu^{2+} and therefore can be exported by TolC and reduces the movement of Cu^{+} into the cytosol, enhancing copper tolerance of S. Typhimurium. *ΔtolC/ΔcueO* has an extremely low copper tolerance, lower than that of *ΔcopA/ΔgolT* which has previously been identified to have extreme sensitivity to copper (Osman *et al.* 2010). This emphasises the importance of detoxifying copper from the periplasm. The main sites of copper toxicity: DNA, proteins and metalloproteins are located within the cytosol. It is possible copper detoxification of the periplasm is important as a preventative measure to reduce copper entry into the cytosol. Alternately copper could have harmful affects upon periplasmic components of S. Typhimurium.

The use of copper as an antimicrobial surface agent provides killing within minutes of adding bacteria to a copper surface (Espirito Santo *et al.* 2011). The speed of bacterial killing indicates that the mechanism of killing is damage to the outer membrane. Current copper toxicity profiles are only present within the cytosol but none of which result in killing within such a short period of time. The exportation of copper from the periplasm may be important to prevent fatal damage to the outer membrane. The novel finding that *ΔtolC/ΔcueO* has

reduced copper tolerance than *ΔcopA/ΔgolT* highlights the importance of periplasmic copper detoxification often over-looked by current copper detoxification models.

It has been shown that under anaerobic conditions a *cueO* mutant of S. Typhimurium has much greater copper toxicity than under aerobic conditions (Achard *et al.* 2010). Under anaerobic conditions copper within the external environment and therefore the periplasm will be present as Cu^{+}. TolC is unable to function under anaerobic conditions possibly due to the lack of Cu^{2+}. As previously stated TolC does not specifically acquire copper from CueO. Once CueO converts Cu^{+} into Cu^{2+}, due to the oxidising environment in the periplasm, Cu^{2+} may not reach TolC before being reduced back into Cu^{+} (Depuydt *et al.* 2012). Both *ΔcueO* under anaerobic conditions and *ΔtolC/ΔcueO* under aerobic conditions have a similar low tolerance to copper. In this study a *ΔtolC/ΔcueO* incubated under aerobic conditions on copper-containing LB agar plates could not survive at 2 mM $CuSO_4$. Achard *et al.* 2010 showed that SL1344 *ΔcueO* grew under anaerobic conditions on copper-containing LB agar plates could not survive at 2 mM $CuSO_4$. The extreme anaerobic copper sensitivity of a *cueO* mutant under anaerobic conditions could be due to TolC not providing copper export in the presence of Cu^{+}. The removal of CueO nullifies the ability of S. Typhimurium to remove copper from the periplasm under anaerobic conditions and would explain the hypersensitive phenotype exhibited by a *cueO* mutant under anaerobic conditions that is not seen under aerobic conditions.

5.12.4 CueP does not provide a role in copper tolerance or homeostasis within S. Typhimurium

Previous work has identified the periplasmic copper binding protein CueP within S. Typhimurium (Pontel and Soncini 2009, Osman *et al.* 2010). It has been reported that a *cueP* mutant has reduced copper tolerance under anaerobic conditions (Pontel and Soncini 2009). TolC was investigated due to clustering of genes encoding TolC dependent RND efflux systems close to genes encoding proteins with sequence similarity to *cueP* across several Gram negative bacteria. Before interactions between CueP and TolC could be investigated, the role of *cueP* in copper tolerance and export was verified. A *cueP* mutant did not show any reduction in copper tolerance compared to SL1344 under aerobic (figures 5.16 and 5.17) or anaerobic growth conditions (figures 5.18 and 5.19) when grown in minimal or rich media, in liquid culture or on solid media. The discrepancy of CueP not providing a role

in anaerobic tolerance and reported by Pontel and Soncini (2009) could be due to difference between the strains of S. Typhimurium used, SL1344 and 14028s.

When a *cueP* mutation was combined with a *tolC* mutant no difference in copper tolerance was seen in comparison to a *tolC* mutant. ICP-MS analysis of metal contents also supported that CueP does not provide a role in copper homeostasis, with a *cueP* mutant accumulating a similar copper quota to SL1344. Osman *et al.* (2010) reported increased copper accumulation upon exposure to higher copper concentrations than used here. A *tolC/cueP* double mutant had a similar copper quota to a *tolC* mutant indicating no additive affect to a *tolC* mutation (table 5.4). From the data gathered in this study it can be concluded that CueP does not provide copper tolerance under aerobic or anaerobic conditions. Recently it has been revealed that CueP is a copper chaperone for $SodC_{II}$ (Osman *et al.* 2013). The conclusion of Osman *et al.* (2013) is in keeping with the tolerance data discovered in this study, that CueP provides a role in supplying copper rather than removing copper from the periplasm for detoxification purposes.

5.12.5 The Ges system does not provide copper homeostasis in S. Typhimurium

After discovering TolC exports copper under aerobic conditions alternate RND efflux systems were investigated in an attempt to identify other exporters capable of removing copper. One RND efflux system identified was the Ges system that had previously been tested for its ability to export a wide range of substrates including: antibiotics, gold and crystal violet (Conroy *et al.* 2010). The *ges* system is regulated by the copper/gold/sulphur responsive regulator GolS, although this study did not include analysis of whether the Ges system is capable of exporting copper (Conroy *et al.* 2010). It is known that the outer membrane factor of the Ges system, GesC, can be replaced by TolC (Nishino *et al.* 2006). A *gesB/gesC* double mutant was tested to examine its copper tolerance under aerobic and anaerobic conditions (figure 5.20). No difference in copper tolerance was identified between *ΔgesB/ΔgesC* and SL1344 when grown under both aerobic and anaerobic conditions. This identified that the Ges system does not contribute to copper tolerance. It is possible that other RND efflux systems which utilise TolC as an outer membrane factor could also export copper such as MdtABC.

5.12.6 Conclusions

The outer membrane exporter TolC aids copper export and tolerance in S. Typhimurium under aerobic but not anaerobic conditions. The reduced copper tolerance of a S. Typhimurium *tolC* mutant is additive to other copper homeostatic proteins such as CueO, CopA and GolT. Although TolC does not function with the CusF like periplasmic copper chaperone CueP. Also, TolC does not export zinc, manganese or cobalt exhibiting specificity towards copper.

Chapter 6

General Conclusions

The use of copper as an antimicrobial agent has been present for several years including its use as fittings within hospitals and food processing factories (Karpanen *et al.* 2012, Borkow and Gabbay 2004). Copper has been identified as a potent antimicrobial agent capable of damaging bacteria by several mechanisms. Prior to this study a recent report identified the use of copper by macrophages as an antimicrobial agent against intracellular *E. coli* (White *et al.* 2009). Little information was known about the use of copper by the immune system and whether its use varied upon the intracellular pathogen. Antimicrobials produced by macrophages and other phagocytes have been studied intensively to gain better understanding of how an immune response is co-ordinated to target intracellular pathogens. Reactive species produced by macrophages have been identified to have significant importance in controlling infection by intracellular pathogens. The interaction of copper with reactive species was investigated to indentify if copper can potentiate the effect of reactive species.

It was discovered by Fenton (1894) that copper and other redox capable metal ions can catalyse Fenton chemistry by the varying oxidation states a metal ion can occupy. This study confirmed previous work that copper can potentiate Fenton chemistry by the addition of copper to hydrogen peroxide. A *ΔcopA/ΔgolT* copper homeostasis mutant contains significantly greater copper quota than wildtype but exhibits the same tolerance to hydrogen peroxide (Osman *et al.* 2010). This confirmed that copper is complexed upon uptake by S. Typhimurium due to the numerous detrimental affects copper can have within the cytosol of S. Typhimurium. Complexed metals have an on/off rate that determines whether the metal stays associated with the protein/ligand or dissociates. The off rate for complexed copper is considered low, but copper can become free for a very brief period of time. For the period that copper is free it could catalyse Fenton chemistry or mediate damage by disrupting metal ligand complexes particularly iron sulphur clusters (Macomber and Imlay 2010). No difference in survival between copper homeostasis mutants and SL1344 to reactive oxygen species stress indicates complexed copper rarely dissociates from its complexed state in response to exposure of S. Typhimurium to these species. Alternatively, the damage caused by hydroxyl radicals is repaired and does not overwhelm cellular repair systems. Complexed copper is highly unlikely to partake in Fenton chemistry therefore the relative quantity of complexed copper does not influence hydrogen peroxide mediated killing. No difference in killing of S. Typhimurium was seen when paraquat and xanthine oxidase were combined

with copper. This study identified copper homeostatic systems in S. Typhimurium protect directly against copper toxicity as opposed to a potentiated affect associated with reactive oxygen species.

Reactive nitrogen species had prior to this study been reported to release complexed copper from the metallothionine MymT in *M. tuberculosis* (Gold *et al.* 2008). Reactive nitrogen species antimicrobial role was investigated in copper homeostasis mutants. *ΔcopA/ΔgolT* had reduced growth in the presence of reactive nitrogen species generators: ASN, GSNO and NOC5/7. This indicated that reactive nitrogen species could be capable of releasing copper in S. Typhimurium due to *ΔcopA/ΔgolT* having reduced growth in comparison to SL1344 S. Typhimurium and/or copper export by CopA and GolT is part of S. Typhimurium reactive nitrogen species defence. β-galactosidase assays identified an increase in expression of both *copA* or *golT* in response to GSNO or NOC 5/7. This implies that copper can be released from bound ligands in S. Typhimurium in response to exposure to reactive nitrogen species. Further work is required to conclude if reactive nitrogen species can liberate copper from within S. Typhimurium. Further β-galactosidase assays could be performed using a *ΔcopA/ΔgolT* strain containing pRS*golT*. β-galactosidase assays cannot be performed with *ΔcopA/ΔgolT* containing pRS*copA* due to expression occurring in response to copper levels within minimal media whereas pRS*golT* expression occurs at a higher copper concentration (Osman *et al.* 2011). As previously stated, *ΔcopA/ΔgolT* contains higher internal copper levels than wildtype. The increased complexed copper levels within *ΔcopA/ΔgolT* provide significantly greater number of targets for reactive nitrogen species to liberate complexed copper from. This could explain why only a small increase in expression of c*opA* and *golT* promoters occurred when S. Typhimurium was exposed to GSNO and NOC 5/7.

This study identified that the addition of copper to peroxynitrite did not influence S. Typhimurium killing. Peroxynitrite is capable of initiating oxidation and nitration of macromolecules without a redox agent. The addition of copper does not provide a functional role in aiding peroxynitrite mediated toxicity. Furthermore as seen with reactive oxygen species killing, peroxynitrite killing is not influenced by intracellular complexed copper levels in S. Typhimurium.

Prior to the start of the study it had been identified that S. Typhimurium is exposed to copper during intracellular infection of Raw 264.7 macrophages and that *ΔcopA/ΔgolT* has reduced

growth in comparison to wildtype in resting macrophages (Osman *et al.* 2011). This study aimed to further understand how copper is utilised as an antimicrobial agent and if reactive nitrogen species contribute to the antimicrobial effects of copper within macrophages. The activation of Raw 264.7 macrophages with IFN-γ prevented replication of S. Typhimurium and *ΔcopA/ΔgolT* had reduced survival than wildtype. This confirmed previous work that activation of macrophages with IFN-γ increases the antimicrobial effectiveness of macrophages and inhibits replication of S. Typhimurium within activated macrophages (Gilberthorpe *et al.* 2007, Vazquez-Torres *et al.* 2008). Nitrite production by macrophages was monitored during competitive infections that confirmed IFN-γ activation increases reactive nitrogen species production; and nitrite levels increase significantly 8 hours post infection when a copper associated antimicrobial response begins against S. Typhimurium (Gordon *et al.* 2005, Erikson *et al.* 2003).

Inhibition of iNOS by the addition of the competitive inhibitor L-NMMA did not change the survival of *ΔcopA/ΔgolT* during infections of IFN-γ activated macrophages. Replication of wildtype S. Typhimurium was seen but *ΔcopA/ΔgolT* could not replicate. Therefore, in the absence of reactive nitrogen species *ΔcopA/ΔgolT* still encounters a copper associated antimicrobial response. These data identified that iNOS activity is not associated with the need for S. Typhimurium copper export during macrophage infection. To identify if macrophages are utilising copper from the surrounding environment for bactericidal activity macrophages were grown under copper limited conditions. Intracellular survival of wildtype and *ΔcopA/ΔgolT* was analysed in macrophages grown in the presence of the copper chelator BCS. *ΔcopA/ΔgolT* survival increased upon BCS addition to DMEM to a similar viable count as wildtype. This identified that bactericidal activity in macrophages is directly associated with environmental copper availability.

Real-time PCR confirmed that during infection of IFN-γ activated and IFN-γ and L-NMMA treated macrophages, *copA* and *golT* are expressed at 12 and 24 hours post infection in intracellular S. Typhimurium consistent with an increased bacterial copper load. S. Typhimurium infection of macrophages pre-treated with IFN-γ and BCS did not exhibit significant changes in *copA* and *golT* expression. Real-time PCR data were therefore consistent with survival data that the requirement for copper export coincides with exposure to copper and not reactive nitrogen species. S. Typhimurium is not exposed to a strong copper antimicrobial response during infection of macrophages treated with BCS that would typically activate *copA* and *golT* expression in the absence of BCS treatment. Studies performed in parallel to this study also confirmed that the addition of BCS significantly

reduces the antimicrobial response of macrophages against S. Typhimurium (Achard *et al.* 2012).

Macrophage copper quotas were analysed after growth in the presence of: IFN-γ, LPS, L-NMMA and BCS. The addition of IFN-γ gave a non-significant increase in copper quota as did LPS in comparison to untreated macrophages. This supported the work of Achard *et al.* (2012) who also identified an increase in expression of copper transporters ATP-7a and Ctr1 in response to LPS exposure. L-NMMA treatment did not influence macrophage copper quota, although BCS treated macrophages had a significantly lower copper quota. This confirmed that BCS treatment lowers copper availability within DMEM and that macrophages accumulate significantly less copper when grown in DMEM containing BCS. Further work is required to identify if macrophages store copper that is mobilised during infection or if a source of copper is required during intracellular infection of macrophages.

When a stress response is triggered within S. Typhimurium, filamentation can occur if replication is inhibited. It has previously been identified that both reactive oxygen species and reactive nitrogen species are capable of initiating filamentation (Schapiro *et al.* 2003, Rosenberger *et al.* 2004). Overnight cultures of S. Typhimurium were grown in the presence of reactive nitrogen species and copper. Filamentation of S. Typhimurium in response to GSNO and NOC5/7 was confirmed in this study. Incubation of *S. typhimurim* in the presence of a $CuSO_4$ concentration (7.5 mM) that inhibits *S. typhimurim* growth rate induces filamentation. It has previously been reported that S. Typhimurium undergoes filamentation within the *Salmonella* containing vacuole (Osman unpublished, Rosenberger *et al.* 2002). Similar levels of filamentous intracellular S. Typhimurium were visualised by fluorescent microscopy of IFN-γ, IFN-γ and L-NMMA treated macrophages. This identified that reactive nitrogen species alone do not trigger filamentation of S. Typhimurium, due to L-NMMA treatment not altering the number of intracellular filamentous S. Typhimurium during infection of macrophages. A significant drop in filamentous intracellular S. Typhimurium, however was seen after BCS treatment of macrophages. This is a novel discovery that a copper antimicrobial response during intracellular infection of macrophages can stimulate filamentation of S. Typhimurium. How copper triggers filamentation is currently unknown and it would be interesting to investigate a possible role for SOS regulator SulA (Mukherjee *et al.* 1998).

Currently there is no known outer membrane transporter of copper within S. Typhimurium. This study investigated TolC as a potential copper exporter. Nishino *et al.* (2007) originally identified that TolC provides a role in copper tolerance, this study built upon this finding. The data obtained in this study confirmed TolC provides a role in copper tolerance under aerobic conditions and a role in copper export at non-toxic copper levels under aerobic conditions. However, TolC does not provide copper tolerance, or reduce the cellular copper load in *S. typhimurim* under anaerobic conditions. This suggests that TolC only exports Cu^{2+} that is predominantly present under aerobic conditions and cannot export Cu^{+} predominantly present under anaerobic conditions. Due to the reduced nature of the cytoplasm it is likely TolC obtains copper from the periplasm where Cu^{2+} is more abundant, by the proposed funnel mechanism, and not from the cytosol where Cu^{+} is abundant (Kim *et al.* 2011). TolC was identified to not provide a role in tolerance or export of zinc, nickel or cobalt showing specificity for copper under aerobic conditions. Future work would be to create inverted vesicles containing purified TolC to identify if TolC exports Cu^{2+} and not Cu^{+} into the inverted vesicles. Also, to identify the contribution to copper export of other RND efflux systems that require TolC.

TolC was also investigated to identify if TolC functions in combination with other copper homeostatic proteins. By the combination of *tolC* mutations with other copper homeostatic mutants it has been concluded that TolC functions independently of the known copper homeostatic proteins in S. Typhimurium such as: CopA, GolT, CueO and CueP. TolC may function in parallel to the actions of CopA and GolT pumping Cu^{+} from the cytosol which if oxidised potentially by CueO then TolC can then export Cu^{2+}. The addition of a *tolC* mutation to strains lacking *copA/golT* and *cueO* gave reduced copper tolerance and survival, indicating separate copper detoxification pathways. Therefore TolC does not require CopA or GolT to provide a source of Cu^{+} or CueO to convert Cu^{+} to Cu^{2+}. TolC still receives Cu^{2+} in the absence of CueO most likely from Cu^{2+} entering the periplasm from the external environment or the oxidation of Cu^{+} exported from the cytosol due to the oxidising nature of the periplasm under aerobic conditions (Beswick *et al.* 1976). But if a *tolC* and *cueO* mutation is combined the *cueO/tolC* double mutant has a hypersensitivity to copper greater than that of a *copA/golT* double mutant. This has identified the importance of copper detoxification within the periplasm of S. Typhimurium; and offers a new copper homeostasis perspective in comparison to the continued focus on the importance of cytosolic copper homeostasis.

In conclusion this study has identified that a macrophage's copper antimicrobial response does not require reactive nitrogen species and is dependent on copper uptaken from the surrounding environment. The requirement for S. Typhimurium copper export in macrophages is associated with a requirement to detoxify copper rather than supply for superoxide dismutase activity to protect against reactive oxygen species. TolC is capable of exporting copper under aerobic conditions contributing to tolerance and detoxification but not under anaerobic conditions.

Bibliography

Abu-Soud, H. M. and D. J. Stuehr (1993). Nitric oxide synthases reveal a role for calmodulin in controlling electron transfer. Proceedings of the National Academy of Sciences of the United States of America 90(22): 10769-10772.

Achard M. E. S., Tree J. T., Holden J.A., Simpfendorfer K. R., Wijburg O. L. C., Strugnell R. A., Schembri M. A., Sweet M. J., Jennings M. P. And McEwan A.G. (2010). The multi-copper ion oxidase CueO of *Salmonella enterica* serovar Typhimurium is required for systemic virulence. Infection and Immunity 78(5): 2312-2319.

Achard M. E. S., Stafford S. L., Bokil N.J., Chartres J., Bernhardt P.V., Schembri M.A., Sweet M.J. and McEwan A.G. (2012). Copper redistribution in murine macrophages in response to *Salmonella* infection.

Adams, P., R. Fowler, N. Kinsella, G. Howell, M. Farris, P. Coote and C. D. O'Connor (2001). Proteomic detection of PhoPQ- and acid-mediated repression of Salmonella motility. Proteomics 1(4): 597-607.

Aleksic, S., F. Heinzerling and J. Bockemuhl (1996). Human infection caused by Salmonellae of subspecies II to VI in Germany, 1977-1992. Zentralbl Bakteriol 283(3): 391-398.

Aljada, A., H. Ghanim and P. Dandona (2002). Translocation of p47phox and activation of NADPH oxidase in mononuclear cells. Methods Molecular Biology 196: 99-103.

Alvarez, B., G. Ferrer-Sueta and R. Radi (1998). Slowing of peroxynitrite decomposition in the presence of mannitol and ethanol. Free Radical Biological Medicine 24(7-8): 1331-1337.

Andreini, C., L. Banci, I. Bertini and A. Rosato (2008). Occurrence of copper proteins through the three domains of life: a bioinformatic approach. Journal of Proteome Research 7(1): 209-216.

Andrews, S. C., D. Shipley, J. N. Keen, J. B. Findlay, P. M. Harrison and J. R. Guest (1992). The haemoglobin-like protein (HMP) of Escherichia coli has ferrisiderophore reductase activity and its C-terminal domain shares homology with ferredoxin NADP+ reductases. FEBS Lettersers 302(3): 247-252.

Arguello, J. M., M. Gonzalez-Guerrero and D. Raimunda (2011). Bacterial transition metal P(1B)-ATPases: transport mechanism and roles in virulence. Biochemistry 50(46): 9940-9949.

Arguello, J. M., A. K. Mandal and S. Mana-Capelli (2003). Heavy metal transport CPx-ATPases from the thermophile Archaeoglobus fulgidus. Annals of the New York Academy of Sciences 986: 212-218.

Askew, S. C., A. R. Butler, F. W. Flitney, G. D. Kemp and I. L. Megson (1995). Chemical mechanisms underlying the vasodilator and platelet anti-aggregating properties of S-nitroso-N-acetyl-DL-penicillamine and S-nitrosoglutathione. Bioorganic & Medicinal Chemistry 3(1): 1-9.

Assari, T. (2006). Chronic Granulomatous Disease; fundamental stages in our understanding of CGD. Medical Immunology 5: 4.

Audia, J. P., C. C. Webb and J. W. Foster (2001). Breaking through the acid barrier: An orchestrated response to proton stress by enteric bacteria. International Journal of Medical Microbiology 291(2): 97-106.

Bagai, I., C. Rensing, N. J. Blackburn and M. M. McEvoy (2008). Direct Metal Transfer between Periplasmic Proteins Identifies a Bacterial Copper Chaperone. Biochemistry 47(44): 11408-11414.

Bailey, A. M., I. T. Paulsen and L. J. Piddock (2008). RamA confers multidrug resistance in *Salmonella enterica* via increased expression of *acrB*, which is inhibited by chlorpromazine. Antimicrobial Agents and Chemotherapy 52(10): 3604-3611.

Balazy, M., P. M. Kaminski, K. Y. Mao, J. Z. Tan and M. S. Wolin (1998). S-nitroglutathione, a product of the reaction between peroxynitrite and glutathione that generates nitric oxide. Journal of Biological Chemistry 273(48): 32009-32015.

Banci, L., I. Bertini, S. Ciofi-Baffoni, M. D'Onofrio, L. Gonnelli, F. C. Marhuenda-Egea and F. J. Ruiz-Duenas (2002). Solution structure of the N-terminal domain of a potential copper-translocating P-type ATPase from Bacillus subtilis in the apo and Cu(I) loaded states. Journal of Molecular Biology 317(3): 415-429.

Bang, I. S., L. M. Liu, A. Vazquez-Torres, M. L. Crouch, J. S. Stamler and F. C. Fang (2006). Maintenance of nitric oxide and redox homeostasis by the Salmonella flavohemoglobin Hmp. Journal of Biological Chemistry 281(38): 28039-28047.

Barber, R. S., R. Braude and K. G. Mitchell (1955). Antibiotic and Copper Supplements for Fattening Pigs. British Journal of Nutrition 9(4): 378-381.

Baucheron, S., C. Mouline, K. Praud, E. Chaslus-Dancla and A. Cloeckaert (2005). TolC but not AcrB is essential for multidrug-resistant *Salmonella enterica* serotype Typhimurium colonization of chicks. Journal of Antimicrobial Chemotherapy 55(5): 707-712.

Bauerfeind, P., R. Garner, B. E. Dunn and H. L. Mobley (1997). Synthesis and activity of Helicobacter pylori urease and catalase at low pH. Gut 40(1): 25-30.

Bearson, B. L., L. Wilson and J. W. Foster (1998). A low pH-inducible, PhoPQ-dependent acid tolerance response protects Salmonella typhimurium against inorganic acid stress. Journal of Bacteriology 180(9): 2409-2417.

Beckman, J. S., H. Ischiropoulos, L. Zhu, M. van der Woerd, C. Smith, J. Chen, J. Harrison, J. C. Martin and M. Tsai (1992). Kinetics of superoxide dismutase- and iron-catalyzed nitration of phenolics by peroxynitrite. Archives of Biochemistry and Biophysics 298(2): 438-445.

Benjamin, N., F. Odriscoll, H. Dougall, C. Duncan, L. Smith, M. Golden and H. Mckenzie (1994). Stomach No Synthesis. Nature 368(6471): 502-502.

Benz, R., E. Maier and I. Gentschev (1993). Tolc of Escherichia-Coli Functions as an Outer-Membrane Channel. Zentralblatt Fur Bakteriologie-International Journal of Medical Microbiology Virology Parasitology and Infectious Diseases 278(2-3): 187-196.

Berg, D. T., A. Gupta, M. A. Richardson, L. A. O'Brien, D. Calnek and B. W. Grinnell (2007). Negative regulation of inducible nitric-oxide synthase expression mediated through transforming growth factor-beta-dependent modulation of transcription factor TCF11. Journal of Biological Chemistry 282(51): 36837-36844.

Bergsbaken, T., S. L. Fink and B. T. Cookson (2009). Pyroptosis: host cell death and inflammation. Nature Reviews Microbiology 7(2): 99-109.

Berlett, B. S. and E. R. Stadtman (1997). Protein oxidation in aging, disease, and oxidative stress. Journal of Biological Chemistry 272(33): 20313-20316.

Bertrand, T., C. Jolivalt, P. Briozzo, E. Caminade, N. Joly, C. Madzak and C. Mougin (2002). Crystal structure of a four-copper laccase complexed with an arylamine: Insights into substrate recognition and correlation with kinetics. Biochemistry 41(23): 7325-7333.

Bessette, P. H., F. Aslund, J. Beckwith and G. Georgiou (1999). Efficient folding of proteins with multiple disulfide bonds in the Escherichia coli cytoplasm. Proceedings of the National Academy of Sciences of the United States of America 96(24): 13703-13708.

Beswick, P. H., G. H. Hall, A. J. Hook, K. Little, D. C. McBrien and K. A. Lott (1976). Copper toxicity: evidence for the conversion of cupric to cuprous copper *in vivo* under anaerobic conditions. Chemico-Biological Interaction 14(3-4): 347-356.

Binet, M. R. B., H. Cruz-Ramos, J. Laver, M. N. Hughes and R. K. Poole (2002). Nitric oxide releases intracellular zinc from prokaryotic metallothionein in Escherichia coli. Fems Microbiology Letters 213(1): 121-126.

Blanc-Potard, A. B., F. Solomon, J. Kayser and E. A. Groisman (1999). The SPI-3 pathogenicity island of *Salmonella enterica*. Journal of Bacteriology 181(3): 998-1004.

Blanco, M., G. Herrera and A. Urios (1995). Increased mutability by oxidative stress in OxyR-deficient Escherichia coli and Salmonella typhimurium cells: clonal occurrence

of the mutants during growth on nonselective media. Mutation Research 346(4): 215-220.

Bodenmiller, D. M. and S. Spiro (2006). The yjeB (nsrR) gene of Escherichia coli encodes a nitric oxide-sensitive transcriptional regulator. Journal of Bacteriology 188(3): 874-881.

Bolotina, V. M., S. Najibi, J. J. Palacino, P. J. Pagano and R. A. Cohen (1994). Nitric-Oxide Directly Activates Calcium-Dependent Potassium Channels in Vascular Smooth-Muscle. Nature 368(6474): 850-853.

Borrelly, G. P., S. A. Rondet, S. Tottey and N. J. Robinson (2004). Chimeras of P-type ATPases and their transcriptional regulators: contributions of a cytosolic amino-terminal domain to metal specificity. Molecular Microbiology 53(1): 217-227.

Boullerne, A. I., K. G. Petry, M. Meynard and M. Geffard (1995). Indirect Evidence for Nitric-Oxide Involvement in Multiple-Sclerosis by Characterization of Circulating Antibodies Directed against Conjugated S-Nitrosocysteine. Journal of Neuroimmunology 60(1-2): 117-124.

Bourret, T. J., M. Song and A. Vazquez-Torres (2009). Codependent and Independent Effects of Nitric Oxide-Mediated Suppression of PhoPQ and Salmonella Pathogenicity Island 2 on Intracellular *Salmonella enterica* Serovar Typhimurium Survival. Infection and Immunity 77(11): 5107-5115.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72 248-254.

Brown, N. F., B. A. Vallance, B. K. Coombes, Y. Valdez, B. A. Coburn and B. B. Finlay (2005). Salmonella pathogenicity island 2 is expressed prior to penetrating the intestine. PLoS Pathogens 1(3): e32.

Bryk, R., P. Griffin and C. Nathan (2000). Peroxynitrite reductase activity of bacterial peroxiredoxins. Nature 407(6801): 211-215.

Buchmeier, N. A. and F. Heffron (1991). Inhibition of macrophage phagosome-lysosome fusion by Salmonella typhimurium. Infection and Immunity 59(7): 2232-2238.

Buchmeier, N. A., S. J. Libby, Y. S. Xu, P. C. Loewen, J. Switala, D. G. Guiney and F. C. Fang (1995). DNA-repair is more important than catalase for Salmonella virulence in mice. Journal of Clinical Investigation 95(3): 1047-1053.

Buchmeier, N. A., C. J. Lipps, M. Y. So and F. Heffron (1993). Recombination-deficient mutants of Salmonella typhimurium are avirulent and sensitive to the oxidative burst of macrophages. Molecular Microbiology 7(6): 933-936.

Buchmeier, N. A., C. J. Lipps, M. Y. H. So and F. Heffron (1993). Recombination-deficient mutants of salmonella-typhimurium are avirulent and sensitive to the oxidative burst of macrophages. Molecular Microbiologyy 7(6): 933-936.

Buckley, A. M., M. A. Webber, S. Cooles, L. P. Randall, R. M. La Ragione, M. J. Woodward and L. J. V. Piddock (2006). The AcrAB-TolC efflux system of *Salmonella enterica* serovar Typhimurium plays a role in pathogenesis. Cellular Microbiology 8(5): 847-856.

Bull, P. C., G. R. Thomas, J. M. Rommens, J. R. Forbes and D. W. Cox (1993). The Wilson Disease Gene Is a Putative Copper Transporting P-Type Atpase Similar to the Menkes Gene. Nature Genetics 5(4): 327-337.

Bullas, L. R. and J. I. Ryu (1983). Salmonella-Typhimurium Lt2 Strains Which Are R- M+ for All 3 Chromosomally Located Systems of DNA Restriction and Modification. Journal of Bacteriology 156(1): 471-474.

Burkey, T. E., K. A. Skjolaas, S. S. Dritz and J. E. Minton (2007). Expression of Toll-like receptors, interleukin 8, macrophage migration inhibitory factor, and osteopontin in tissues from pigs challenged with *Salmonella enterica* serovar Typhimurium or serovar Choleraesuis. Veterinary Immunology and Immunopathology 115(3-4): 309-319.

Calabrese, V., G. Scapagnini, A. Ravagna, R. Bella, R. Foresti, T. E. Bates, A. M. G. Stella and G. Pennisi (2002). Nitric oxide synthase is present in the cerebrospinal fluid of patients with active multiple sclerosis and is associated with increases in cerebrospinal fluid protein nitrotyrosine and S-nitrosothiols and with changes in glutathione levels. Journal of Neuroscience Research 70(4): 580-587.

Casareno, R. L., D. Waggoner and J. D. Gitlin (1998). The copper chaperone CCS directly interacts with copper/zinc superoxide dismutase. Journal of Biology and Chemistry273(37): 23625-23628.

Castelli, M. E., E. Garcia Vescovi and F. C. Soncini (2000). The phosphatase activity is the target for Mg2+ regulation of the sensor protein PhoQ in Salmonella. Journal of Biology and Chemistry 275(30): 22948-22954.

Cavet, J. S., W. Meng, M. A. Pennella, R. J. Appelhoff, D. P. Giedroc and N. J. Robinson (2002). A nickel-cobalt-sensing ArsR-SmtB family repressor. Contributions of cytosol and effector binding sites to metal selectivity. Journal of Biology and Chemistry 277(41): 38441-38448.

Chakravortty, D., I. Hansen-Wester and M. Hensel (2002). Salmonella pathogenicity island 2 mediates protection of intracellular Salmonella from reactive nitrogen intermediates. The Journal of Experimental Medicine 195(9): 1155-1166.

Chang, A. C. and S. N. Cohen (1978). Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. Journal of Bacteriology 134(3): 1141-1156.

Changela, A., K. Chen, Y. Xue, J. Holschen, C. E. Outten, T. V. O'Halloran and A. Mondragon (2003). Molecular basis of metal-ion selectivity and zeptomolar sensitivity by CueR. Science 301(5638): 1383-1387.

Charnsilpa, W., R. Takhampunya, T. P. Endy, M. P. Mammen, Jr., D. H. Libraty and S. Ubol (2005). Nitric oxide radical suppresses replication of wild-type dengue 2 viruses in vitro. Journal of Medical Virology 77(1): 89-95.

Chaturvedi, K. S., C. S. Hung, J. R. Crowley, A. E. Stapleton and J. P. Henderson (2012). The siderophore yersiniabactin binds copper to protect pathogens during infection. Nature Chemical Biology 8(8): 731-736.

Checa, S. K., M. Espariz, M. E. P. Audero, P. E. Botta, S. V. Spinelli and F. C. Soncini (2007). Bacterial sensing of and resistance to gold salts. Molecular Microbiology 63(5): 1307-1318.

Chelly, J., Z. Tumer, T. Tonnesen, A. Petterson, Y. Ishikawa-Brush, N. Tommerup, N. Horn and A. P. Monaco (1993). Isolation of a candidate gene for Menkes disease that encodes a potential heavy metal binding protein. Nature Genetics 3(1): 14-19.

Chen, C. Y., J. L. Hsieh, S. Silver, G. Endo and C. C. Huang (2008). Interactions between two MerR regulators and three operator/promoter regions in the mercury resistance module of Bacillus megaterium. Bioscience Biotechnology and Biochemistry 72(9): 2403-2410.

Cherepanov, P. P. and W. Wackernagel (1995). Gene disruption in Escherichia coli: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. Gene 158(1): 9-14.

Chesney, J. A., J. W. Eaton and J. R. Mahoney, Jr. (1996). Bacterial glutathione: a sacrificial defense against chlorine compounds. Journal of Bacteriology 178(7): 2131-2135.

Christman, M. F., R. W. Morgan, F. S. Jacobson and B. N. Ames (1985). Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in Salmonella typhimurium. Cell 41(3): 753-762.

Chung, J., D. J. Haile and M. Wessling-Resnick (2004). Copper-induced ferroportin-1 expression in J774 macrophages is associated with increased iron efflux. Proceedings of the National Academy of Sciences of the United States of America 101(9): 2700-2705.

Clement, M. V. and I. Stamenkovic (1996). Superoxide anion is a natural inhibitor of FAS-mediated cell death. EMBO J 15(2): 216-225.

Cobine, P., W. A. Wickramasinghe, M. D. Harrison, T. Weber, M. Solioz and C. T. Dameron (1999). The Enterococcus hirae copper chaperone CopZ delivers copper(I) to the CopY repressor. FEBS Letters 445(1): 27-30.

Conroy, O., E. H. Kim, M. M. McEvoy and C. Rensing (2010). Differing ability to transport nonmetal substrates by two RND-type metal exporters. FEMS Microbiol Lett 308(2): 115-122.

Coombes, B. K., B. A. Coburn, A. A. Potter, S. Gomis, K. Mirakhur, Y. Li and B. B. Finlay (2005). Analysis of the contribution of Salmonella pathogenicity islands 1 and 2 to enteric disease progression using a novel bovine ileal loop model and a murine model of infectious enterocolitis. Infection and Immunity 73(11): 7161-7169.

Cooper, C. E. (1999). Nitric oxide and iron proteins. Biochimica et Biophysica Acta 1411(2-3): 290-309.

Craig, M. and J. M. Slauch (2009). Phagocytic superoxide specifically damages an extracytoplasmic target to inhibit or kill Salmonella. PLoS One 4(3): e4975.

Crawford, M. J. and D. E. Goldberg (1998). Role for the Salmonella flavohemoglobin in protection from nitric oxide. Journal of Biology and Chemistry273(20): 12543-12547.

Crocker, P. R., J. M. Blackwell and D. J. Bradley (1984). Expression of the natural resistance gene Lsh in resident liver macrophages. Infection and Immunity 43(3): 1033-1040.

Crosby, J. A. and S. C. Kachlany (2007). TdeA, a TolC-like protein required for toxin and drug export in Aggregatibacter (Actinobacillus) actinomycetemcomitans. Gene 388(1-2): 83-92.

Cruz-Ramos, H., J. Crack, G. G. Wu, M. N. Hughes, C. Scott, A. J. Thomson, J. Green and R. K. Poole (2002). NO sensing by FNR: regulation of the Escherichia coli NO-detoxifying flavohaemoglobin, Hmp. Embo Journal 21(13): 3235-3244.

D'Autreaux, B., N. P. Tucker, R. Dixon and S. Spiro (2005). A non-haem iron centre in the transcription factor NorR senses nitric oxide. Nature 437(7059): 769-772.

Darnell, J. E., I. M. Kerr and G. R. Stark (1994). Jak-Stat Pathways and Transcriptional Activation in Response to Ifns and Other Extracellular Signaling Proteins. Science 264(5164): 1415-1421.

Darwin, A. J. and V. Stewart (1995). Nitrate and Nitrite Regulation of the Fnr-Dependent Aeg-46.5 Promoter of Escherichia-Coli K-12 Is Mediated by Competition between Homologous Response Regulators (Narl and Narp) for a Common DNA-Binding Site. Journal of Molecular Biology 251(1): 15-29.

Datsenko, K. A. and B. L. Wanner (2000). One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proceedings of the National Academy of Sciences of the United States of America 97(12): 6640-6645.

Deiwick, J., T. Nikolaus, J. E. Shea, C. Gleeson, D. W. Holden and M. Hensel (1998). Mutations in Salmonella pathogenicity island 2 (SP12) genes affecting transcription of SPI1 genes and resistance to antimicrobial agents. Journal of Bacteriology 180(18): 4775-4780.

Deiwick, J., C. Rappl, S. Stender, P. R. Jungblut and M. Hensel (2002). Proteomic approaches to Salmonella Pathogenicity Island 2 encoded proteins and the SsrAB regulon. Proteomics 2(6): 792-799.

Denicola, A., B. A. Freeman, M. Trujillo and R. Radi (1996). Peroxynitrite reaction with carbon dioxide/bicarbonate: Kinetics and influence on peroxynitrite-mediated oxidations. Archives of Biochemistry and Biophysics 333(1): 49-58.

Depuydt, M., S. E. Leonard, D. Vertommen, K. Denoncin, P. Morsomme, K. Wahni, J. Messens, K. S. Carroll and J. F. Collet (2009). A Periplasmic Reducing System Protects Single Cysteine Residues from Oxidation. Science 326(5956): 1109-1111.

Dhamdhere, G. and H. I. Zgurskaya (2010). Metabolic shutdown in Escherichia coli cells lacking the outer membrane channel TolC. Molecular Microbiology 77(3): 743-754.

Djoko, K. Y., J. A. Franiek, J. L. Edwards, M. L. Falsetta, S. P. Kidd, A. J. Potter, N. H. Chen, M. A. Apicella, M. P. Jennings and A. G. McEwan (2012). Phenotypic Characterization of a copA Mutant of Neisseria gonorrhoeae Identifies a Link between Copper and Nitrosative Stress. Infection and Immunity 80(3): 1065-1071.

Eichelberg, K. and J. E. Galan (1999). Differential regulation of Salmonella typhimurium type III secreted proteins by pathogenicity island 1 (SPI-1)-encoded transcriptional activators InvF and HilA. Infection and Immunity 67(8): 4099-4105.

Eisenstark, A., M. J. Calcutt, M. Becker-Hapak and A. Ivanova (1996). Role of Escherichia coli rpoS and associated genes in defense against oxidative damage. Free Radical Biology & Medicine 21(7): 975-993.

Eriksson, S., S. Lucchini, A. Thompson, M. Rhen and J. C. D. Hinton (2003). Unravelling the biology of macrophage infection by gene expression profiling of intracellular *Salmonella enterica*. Molecular Microbiology 47(1): 103-118.

Espariz, M., S. K. Checa, M. E. Perez Audero, L. B. Pontel and F. C. Soncini (2007). Dissecting the Salmonella response to copper. Microbiology 153: 2989-2997.

Eswarappa, S. M., V. D. Negi, S. Chakraborty, B. K. C. Sagar and D. Chakravortty (2010). Division of the Salmonella-Containing Vacuole and Depletion of Acidic Lysosomes in Salmonella-Infected Host Cells Are Novel Strategies of *Salmonella enterica* To Avoid Lysosomes. Infection and Immunity 78(1): 68-79.

Eu, J. P., L. Liu, M. Zeng and J. S. Stamler (2000). An apoptotic model for nitrosative stress. Biochemistry 39(5): 1040-1047.

Fang, F. C., A. Vazquez-Torres and Y. Xu (1997). The transcriptional regulator SoxS is required for resistance of Salmonella typhimurium to paraquat but not for virulence in mice. Infection and Immunity 65(12): 5371-5375.

Felix, A. and R. M. Pitt (1934). A new antigen of B typhosus - Its relation to virulence and to active and passive immunisation. Lancet 2: 186-191.

Fenton, H. J. H. (1894). LXXIII.-Oxidation of tartaric acid in presence of iron. Journal of the Chemical Society, Transactions 65(0): 899-910.

Ferhat, M., D. Atlan, A. Vianney, J. C. Lazzaroni, P. Doublet and C. Gilbert (2009). The TolC protein of Legionella pneumophila plays a major role in multi-drug resistance and the early steps of host invasion. PLoS One 4(11): e7732.

Filenko, N., S. Spiro, D. F. Browning, D. Squire, T. W. Overton, J. Cole and C. Constantinidou (2007). The NsrR regulon of Escherichia coli K-12 includes genes encoding the hybrid cluster protein and the periplasmic, respiratory nitrite reductase. Journal of Bacteriology 189(12): 4410-4417.

Fink, R. C., M. R. Evans, S. Porwollik, A. Vazquez-Torres, J. Jones-Carson, B. Troxell, S. J. Libby, M. McClelland and H. M. Hassan (2007). FNR is a global regulator of virulence and anaerobic metabolism in *Salmonella enterica* serovar Typhimurium (ATCC 14028s). Journal of Bacteriology 189(6): 2262-2273.

Finlay, B. B., S. Ruschkowski and S. Dedhar (1991). Cytoskeletal rearrangements accompanying salmonella entry into epithelial cells. Journal of Cell Science 99: 283-296.

Flatley, J., J. Barrett, S. T. Pullan, M. N. Hughes, J. Green and R. K. Poole (2005). Transcriptional responses of Escherichia coli to S-nitrosoglutathione under defined chemostat conditions reveal major changes in methionine biosynthesis. Journal of Biological Chemistry 280(11): 10065-10072.

Foster, A. W. and N. J. Robinson (2011). Promiscuity and preferences of metallothioneins: the cell rules. Bmc Biology 9.

Foster, J. W. and H. K. Hall (1990). Adaptive Acidification Tolerance Response of Salmonella-Typhimurium. Journal of Bacteriology 172(2): 771-778.

Foster, J. W. and H. K. Hall (1990). Adaptive acidification tolerance response of Salmonella typhimurium. Journal of Bacteriology 172(2): 771-778.

Foster, J. W. and M. Moreno (1999). Inducible acid tolerance mechanisms in enteric bacteria. Novartis Found Symp 221: 55-69; discussion 70-54.

Fralick, J. A. (1996). Evidence that TolC is required for functioning of the Mar/AcrAB efflux pump of Escherichia coli. Journal of Bacteriology 178(19): 5803-5805.

Franchi, L., J. Stoolman, T. D. Kanneganti, A. Verma, R. Ramphal and G. Nunez (2007). Critical role for Ipaf in Pseudomonas aeruginosa-induced caspase-1 activation. European Journal of Immunology 37(11): 3030-3039.

Franchi, L., J. Stoolman, T. D. Kanneganti, A. Verma, R. Rarnphal and G. Nunez (2007). Critical role for Ipaf in Pseudomonas aeruginosa-induced caspase-1 activation. European Journal of Immunology 37(11): 3030-3039.

Francis, C. L., T. A. Ryan, B. D. Jones, S. J. Smith and S. Falkow (1993). Ruffles Induced by Salmonella and Other Stimuli Direct Macropinocytosis of Bacteria. Nature 364(6438): 639-642.

Francis, M. S. and C. J. Thomas (1997). Mutants in the CtpA copper transporting P-type ATPase reduce virulence of Listeria monocytogenes. Microbial Pathogenesis 22(2): 67-78.

Fu, Y. and J. E. Galan (1999). A salmonella protein antagonizes Rac-1 and Cdc42 to mediate host-cell recovery after bacterial invasion. Nature 401(6750): 293-297.

Galan, J. E. and A. Collmer (1999). Type III secretion machines: bacterial devices for protein delivery into host cells. Science 284(5418): 1322-1328.

Garthwaite, J., S. L. Charles and R. Chess-Williams (1988). Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. Nature 336(6197): 385-388.

Gerschman, R., D. L. Gilbert, S. W. Nye, P. Dwyer and W. O. Fenn (1954). Oxygen poisoning and x-irradiation: a mechanism in common. Science 119(3097): 623-626.

Gharieb, M. M. and G. M. Gadd (2004). Role of glutathione in detoxification of metal(loid)s by Saccharomyces cerevisiae. Biometals 17(2): 183-188.

Gil, H., G. J. Platz, C. A. Forestal, M. Monfett, C. S. Bakshi, T. J. Sellati, M. B. Furie, J. L. Benach and D. G. Thanassi (2006). Deletion of TolC orthologs in Francisella tularensis identifies roles in multidrug resistance and virulence. Proceedings of the National Academy of Sciences of the United States of America 103(34): 12897-12902.

Gilberthorpe, N. J., M. E. Lee, T. M. Stevanin, R. C. Read and R. K. Poole (2007). NsrR: a key regulator circumventing *Salmonella enterica* serovar Typhimurium oxidative and nitrosative stress in vitro and in IFN-gamma-stimulated J774.2 macrophages. Microbiology 153(Pt 6): 1756-1771.

Giraud, E., A. Cloeckaert, D. Kerboeuf and E. Chaslus-Dancla (2000). Evidence for active efflux as the primary mechanism of resistance to ciprofloxacin in *Salmonella enterica* serovar typhimurium. Antimicrobial Agents and Chemotherapy 44(5): 1223-1228.

Girotti, A. W. (1985). Mechanisms of lipid peroxidation. Journal of Free Radical Biology & Medicine 1(2): 87-95.

Gold, B., H. Deng, R. Bryk, D. Vargas, D. Eliezer, J. Roberts, X. Jiang and C. Nathan (2008). Identification of a copper-binding metallothionein in pathogenic mycobacteria. Nature Chemical Biology 4(10): 609-616.

Gonzalez-Flecha, B. and B. Demple (1995). Metabolic sources of hydrogen peroxide in aerobically growing Escherichia coli. Journal of Biology and Chemistry270(23): 13681-13687.

Gonzalez-Guerrero, M., D. Raimunda, X. Cheng and J. M. Arguello (2010). Distinct functional roles of homologous Cu plus efflux ATPases in Pseudomonas aeruginosa. Molecular Microbiologyy 78(5): 1246-1258.

Gordon, M. A., D. L. Jack, D. H. Dockrell, M. E. Lee and R. C. Read (2005). Gamma interferon enhances internalization and early nonoxidative killing of *Salmonella enterica* serovar typhimurium by human macrophages and modifies cytokine responses. Infection and Immunity 73(6): 3445-3452.

Gorren, A. C., A. Schrammel, K. Schmidt and B. Mayer (1996). Decomposition of S-nitrosoglutathione in the presence of copper ions and glutathione. Archives of Biochemistry and Biophysics 330(2): 219-228.

Goswami, M., S. H. Mangoli and N. Jawali (2006). Involvement of reactive oxygen species in the action of ciprofloxacin against Escherichia coli. Antimicrobial Agents and Chemotherapy 50(3): 949-954.

Goswami, T., A. Bhattacharjee, P. Babal, S. Searle, E. Moore, M. Li and J. M. Blackwell (2001). Natural-resistance-associated macrophage protein 1 is an H+/bivalent cation antiporter. Biochemical Journal 354(Pt 3): 511-519.

Gotuzzo, E., J. G. Morris, L. Benavente, P. K. Wood, O. Levine, R. E. Black and M. M. Levine (1987). Association between Specific Plasmids and Relapse in Typhoid-Fever. Journal of Clinical Microbiology 25(9): 1779-1781.

Gow, P. J., R. A. Smallwood, P. W. Angus, A. L. Smith, A. J. Wall and R. B. Sewell (2000). Diagnosis of Wilson's disease: an experience over three decades. Gut 46(3): 415-419.

Graham, J. E. and J. E. Clark-Curtiss (1999). Identification of Mycobacterium tuberculosis RNAs synthesized in response to phagocytosis by human macrophages by selective capture of transcribed sequences (SCOTS). Proceedings of the National Academy of Sciences of the United States of America 96(20): 11554-11559.

Grant, A. J., G. L. Foster, T. J. McKinley, S. P. Brown, S. Clare, D. J. Maskell and P. Mastroeni (2009). Bacterial growth rate and host factors as determinants of intracellular bacterial distributions in systemic *Salmonella enterica* infections. Infection and Immunity 77(12): 5608-5611.

Grass, G. and C. Rensing (2001). Genes involved in copper homeostasis in Escherichia coli. Journal of Bacteriology 183(6): 2145-2147.

Grupta A., Phung L.T., Taylor D. E., and Silver S. (2001). Diversity of silver resistance gene in IncH incompatibility group plasmids. Microbiology 147(12): 3393-3402.

Hamza, I., J. Prohaska and J. D. Gitlin (2003). Essential role for Atox1 in the copper-mediated intracellular trafficking of the Menkes ATPase. Proceedings of the National Academy of Sciences of the United States of America 100(3): 1215-1220.

Hansen-Wester, I., D. Chakravortty and M. Hensel (2004). Functional transfer of Salmonella pathogenicity island 2 to Salmonella bongori and Escherichia coli. Infection and Immunity 72(5): 2879-2888.

Hardt, W. D., L. M. Chen, K. E. Schuebel, X. R. Bustelo and J. E. Galan (1998). S. typhimurium encodes an activator of Rho GTPases that induces membrane ruffling and nuclear responses in host cells. Cell 93(5): 815-826.

Harris, E. D. (2001). Copper homeostasis: the role of cellular transporters. Nutrition Reviews 59(9): 281-285.

Hayward, R. D. and V. Koronakis (1999). Direct nucleation and bundling of actin by the SipC protein of invasive Salmonella. EMBO J 18(18): 4926-4934.

Hebrard, M., J. P. Viala, S. Meresse, F. Barras and L. Aussel (2009). Redundant hydrogen peroxide scavengers contribute to Salmonella virulence and oxidative stress resistance. Journal of Bacteriology 191(14): 4605-4614.

Helaine, S., J. A. Thompson, K. G. Watson, M. Liu, C. Boyle and D. W. Holden (2010). Dynamics of intracellular bacterial replication at the single cell level. Proceedings of the National Academy of Sciences of the United States of America 107(8): 3746-3751.

Helbig, K., C. Bleuel, G. J. Krauss and D. H. Nies (2008). Glutathione and transition-metal homeostasis in Escherichia coli. Journal of Bacteriology 190(15): 5431-5438.

Henderson, P. T., J. C. Delaney, F. Gu, S. R. Tannenbaum and J. M. Essigmann (2002). Oxidation of 7,8-dihydro-8-oxoguanine affords lesions that are potent sources of replication errors *in vivo*. Biochemistry 41(3): 914-921.

Henderson, P. T., J. C. Delaney, F. Gu, S. R. Tannenbaum and J. M. Essigmann (2002). Oxidation of 7,8-dihydro-8-oxoguanine affords lesions that are potent sources of replication errors *in vivo*. Biochemistry 41(3): 914-921.

Hernandez-Montes, G., J. M. Arguello and B. Valderrama (2012). Evolution and diversity of periplasmic proteins involved in copper homeostasis in gamma proteobacteria. BMC Microbiologyogy 12.

Hernandez-Urzua, E., D. S. Zamorano-Sanchez, J. Ponce-Coria, E. Morett, S. Grogan, R. K. Poole and J. Membrillo-Hernandez (2007). Multiple regulators of the Flavohaemoglobin (hmp) gene of *Salmonella enterica* serovar Typhimurium include RamA, a transcriptional regulator conferring the multidrug resistance phenotype. Archives of Microbiology 187(1): 67-77.

Hibbs, J. B., R. R. Taintor and Z. Vavrin (1987). Macrophage Cytotoxicity - Role for L-Arginine Deiminase and Imino-Nitrogen Oxidation to Nitrite. Science 235(4787): 473-476.

Ho, T. D., N. Figueroa-Bossi, M. Wang, S. Uzzau, L. Bossi and J. M. Slauch (2002). Identification of GtgE, a novel virulence factor encoded on the Gifsy-2 bacteriophage of *Salmonella enterica* serovar Typhimurium. Journal of Bacteriology 184(19): 5234-5239.

Hobbie, S., L. M. Chen, R. J. Davis and J. E. Galan (1997). Involvement of mitogen-activated protein kinase pathways in the nuclear responses and cytokine production induced by Salmonella typhimurium in cultured intestinal epithelial cells. The Journal of Immunology 159(11): 5550-5559.

Holmgren, A. (1989). Thioredoxin and glutaredoxin systems. Journal of Biology and Chemistry 264(24): 13963-13966.

Horiyama, T., A. Yamaguchi and K. Nishino (2010). TolC dependency of multidrug efflux systems in *Salmonella enterica* serovar Typhimurium. Journal of Antimicrobial Chemotherapy 65(7): 1372-1376.

Hou, Z. J., S. Narindrasorasak, B. Bhushan, B. Sarkar and B. Mitra (2001). Functional analysis of chimeric proteins of the Wilson Cu(I)-ATPase (ATP7B) and ZntA, a Pb(II)/Zn(II)/Cd(II)-ATPase from Escherichia coli. Journal of Biology and Chemistry 276(44): 40858-40863.

Hu, L. T. and H. L. Mobley (1990). Purification and N-terminal analysis of urease from Helicobacter pylori. Infection and Immunity 58(4): 992-998.

Hurshman, A. R. and M. A. Marletta (1995). Nitric oxide complexes of inducible nitric oxide synthase: spectral characterization and effect on catalytic activity. Biochemistry 34(16): 5627-5634.

Ibanez, M. M., S. Cerminati, S. K. Checa and F. C. Soncini (2013). Dissecting the metal selectivity of MerR monovalent metal ion sensors in Salmonella. Journal of Bacteriology 195(13): 3084-3092.

Ikeda, R., T. Shinoda, T. Morita and E. S. Jacobson (1993). Characterization of a phenol oxidase from Cryptococcus neoformans var. neoformans. Microbiology and Immunology 37(10): 759-764.

Imlay, K. R. C. and J. A. Imlay (1996). Cloning and analysis of sodC, encoding the copper-zinc superoxide dismutase of Escherichia coli. Journal of Bacteriology 178(9): 2564-2571.

Inoue, K., T. Akaike, Y. Miyamoto, T. Okamoto, T. Sawa, M. Otagiri, S. Suzuki, T. Yoshimura and H. Maeda (1999). Nitrosothiol formation catalyzed by ceruloplasmin. Implication for cytoprotective mechanism *in vivo*. Journal of Biology and Chemistry 274(38): 27069-27075.

Irving, H. and R. J. P. Williams (1948). Order of Stability of Metal Complexes. Nature 162(4123): 746-747.

Ishibashi, Y. and T. Arai (1995). Salmonella typhi does not inhibit phagosome-lysosome fusion in human monocyte-derived macrophages. FEMS Immunology and Medical Microbiology12(1): 55-61.

Iyengar, R., D. J. Stuehr and M. A. Marletta (1987). Macrophage Synthesis of Nitrite, Nitrate, and N-Nitrosamines - Precursors and Role of the Respiratory Burst. Proceedings of the National Academy of Sciences of the United States of America 84(18): 6369-6373.

Jain, S., S. A. Bidol, J. L. Austin, E. Berl, F. Elson, M. Lemaile-Williams, M. Deasy, 3rd, M. E. Moll, V. Rea, J. D. Vojdani, P. A. Yu, R. M. Hoekstra, C. R. Braden and M. F. Lynch (2009). Multistate outbreak of Salmonella Typhimurium and Saintpaul infections associated with unpasteurized orange juice--United States, 2005. Clinical Infectious Diseases 48(8): 1065-1071.

Jepson, M. A., H. B. Schlecht and C. B. Collares-Buzato (2000). Localization of dysfunctional tight junctions in *Salmonella enterica* serovar typhimurium-infected epithelial layers. Infection and Immunity 68(12): 7202-7208.

Jin, Y. H., P. E. Dunlap, S. J. McBride, H. Al-Refai, P. R. Bushel and J. H. Freedman (2008). Global transcriptome and deletome profiles of yeast exposed to transition metals. PLoS Geneticsics 4(4): e1000053.

Jones, B. D., N. Ghori and S. Falkow (1994). Salmonella typhimurium initiates murine infection by penetrating and destroying the specialized epithelial M cells of the Peyer's patches. The Journal of Experimental Medicine 180(1): 15-23.

Jones, D. G. and N. F. Suttle (1981). Some effects of copper deficiency on leucocyte function in sheep and cattle. Research in Veterinary Science 31(2): 151-156.

Justino, M. C., C. C. Almeida, M. Teixeira and L. M. Saraiva (2007). Escherichia coli di-iron YtfE protein is necessary for the repair of stress-damaged iron-sulfur clusters. Journal of Biology and Chemistry 282(14): 10352-10359.

Kalupahana, R. S., P. Mastroeni, D. Maskell and B. A. Blacklaws (2005). Activation of murine dendritic cells and macrophages induced by *Salmonella enterica* serovar Typhimurium. Immunology 115(4): 462-472.

Kamal, N. and W. M. Shafer (2010). Biologic activities of the TolC-like protein of Neisseria meningitidis as assessed by functional complementation in Escherichia coli. Antimicrobial Agents and Chemotherapy 54(1): 506-508.

Kamijo, R., H. Harada, T. Matsuyama, M. Bosland, J. Gerecitano, D. Shapiro, J. Le, S. I. Koh, T. Kimura, S. J. Green and et al. (1994). Requirement for transcription factor IRF-1 in NO synthase induction in macrophages. Science 263(5153): 1612-1615.

Kauffmann, F. (1966). [On the history of salmonella research]. Zentralbl Bakteriol Original 201(1): 44-48.

Keller, L. H., C. E. Benson, K. Krotec and R. J. Eckroade (1995). Salmonella enteritidis colonization of the reproductive tract and forming and freshly laid eggs of chickens. Infection and Immunity 63(7): 2443-2449.

Khoroshilova, N., H. Beinert and P. J. Kiley (1995). Association of a polynuclear iron-sulfur center with a mutant FNR protein enhances DNA binding. Proceedings of the National Academy of Sciences of the United States of America 92(7): 2499-2503.

Kidd S. P. Djoko K.Y., NG J., Argente M.P., Jennings M.P. and McEwan A. G (2011). A novel nickel responsive MerR-like regulator, NimR, from Haemophilus influenzae. Metallomics 3(10): 1009-1018.

Kim, E. H., D. H. Nies, M. M. McEvoy and C. Rensing (2011). Switch or funnel: how RND-type transport systems control periplasmic metal homeostasis. Journal of Bacteriology 193(10): 2381-2387.

Kim, H., H. Y. Son, S. M. Bailey and J. Lee (2009). Deletion of hepatic Ctr1 reveals its function in copper acquisition and compensatory mechanisms for copper homeostasis. American Journal of Physiology - Gastrointestinal and Liver Physiology 296(2): G356-364.

Kim, H. W., Q. Chan, S. E. Afton, J. A. Caruso, B. Lai, N. L. Weintraub and Z. Qin (2012). Human macrophage ATP7A is localized in the trans-Golgi apparatus, controls intracellular copper levels, and mediates macrophage responses to dermal wounds. Inflammation 35(1): 167-175.

Kim, J. S., M. H. Kim, M. H. Joe, S. S. Song, I. S. Lee and S. Y. Choi (2002). The sctR of *Salmonella enterica* serova Typhimurium encoding a homologue of MerR protein is involved in the copper-responsive regulation of cuiD. FEMS Microbiol Letters 210(1): 99-103.

Kirby, A. C., U. Yrlid and M. J. Wick (2002). The innate immune response differs in primary and secondary Salmonella infection. The Journal of Immunology 169(8): 4450-4459.

Klebanoff, S. J. (1967). Iodination of bacteria: a bactericidal mechanism. The Journal of Experimental Medicine 126(6): 1063-1078.

Kohbata, S., H. Yokoyama and E. Yabuuchi (1986). Cytopathogenic effect of Salmonella typhi GIFU 10007 on M cells of murine ileal Peyer's patches in ligated ileal loops: an ultrastructural study. Microbiology and Immunology 30(12): 1225-1237.

Kohen, R. and M. Chevion (1985). Transition metals potentiate paraquat toxicity. Free radical research communications 1(2): 79-88.

Koronakis, V., J. Li, E. Koronakis and K. Stauffer (1997). Structure of TolC, the outer membrane component of the bacterial type I efflux system, derived from two-dimensional crystals. Molecular Microbiology 23(3): 617-626.

Koronakis, V., A. Sharff, E. Koronakis, B. Luisi and C. Hughes (2000). Crystal structure of the bacterial membrane protein TolC central to multidrug efflux and protein export. Nature 405(6789): 914-919.

Koshkin, V. and E. Pick (1993). Generation of superoxide by purified and relipidated cytochrome b559 in the absence of cytosolic activators. FEBS Letters 327(1): 57-62.

Krell, T., J. Lacal, A. Busch, H. Silva-Jimenez, M. E. Guazzaroni and J. L. Ramos (2010). Bacterial sensor kinases: diversity in the recognition of environmental signals. Annual Reviews Microbiology 64: 539-559.

Kubori, T. and J. E. Galan (2003). Temporal regulation of salmonella virulence effector function by proteasome-dependent protein degradation. Cell 115(3): 333-342.

Kuhn, D. E., B. D. Baker, W. P. Lafuse and B. S. Zwilling (1999). Differential iron transport into phagosomes isolated from the RAW264.7 macrophage cell lines transfected with Nramp1Gly169 or Nramp1Asp169. Journal of Leukocyte Biology 66(1): 113-119.

Kulasekara, H. D., I. Ventre, B. R. Kulasekara, A. Lazdunski, A. Filloux and S. Lory (2005). A novel two-component system controls the expression of Pseudomonas aeruginosa fimbrial cup genes. Molecular Microbiology 55(2): 368-380.

Lancaster, J. R., Jr. (1997). A tutorial on the diffusibility and reactivity of free nitric oxide. Nitric Oxide 1(1): 18-30.

Lara-Tejero, M., F. S. Sutterwala, Y. Ogura, E. P. Grant, J. Bertin, A. J. Coyle, R. A. Flavell and J. E. Galan (2006). Role of the caspase-1 inflammasome in Salmonella typhimurium pathogenesis. The Journal of Experimental Medicine 203(6): 1407-1412.

Lazazzera, B. A., D. M. Bates and P. J. Kiley (1993). The activity of the Escherichia coli transcription factor FNR is regulated by a change in oligomeric state. Genes & Development 7(10): 1993-2005.

Lee S. M., Grass G., Rensing C., Barrett S. R., Yates C. J. D., Stoyanov J. V., and Brown N. L. (2002). The Pco proteins are involved in periplasmic copper handling in *Escherichia coli*. Biochemical and Biophysical research communications 295(3): 616-620.

Lemire J.A., Harrison J.J., and Turner R.J. (2013). Antimicrobial activity of metals: mechanisms, molecular targets and applications. Nature reviews Microbiology 11(6): 371-384.

Levine, R. L., L. Mosoni, B. S. Berlett and E. R. Stadtman (1996). Methionine residues as endogenous antioxidants in proteins. Proceedings of the National Academy of Sciences of the United States of America 93(26): 15036-15040.

Lewinson, O., A. T. Lee and D. C. Rees (2009). A P-type ATPase importer that discriminates between essential and toxic transition metals. Proceedings of the National Academy of Sciences of the United States of America 106(12): 4677-4682.

Li, W. R., X. B. Xie, Q. S. Shi, H. Y. Zeng, Y. S. Ou-Yang and Y. B. Chen (2010). Antibacterial activity and mechanism of silver nanoparticles on Escherichia coli. Applied Microbiology and Biotechnology 85(4): 1115-1122.

Lin, S. J. and V. C. Culotta (1995). The ATX1 gene of Saccharomyces cerevisiae encodes a small metal homeostasis factor that protects cells against reactive oxygen toxicity. Proceedings of the National Academy of Sciences of the United States of America 92(9): 3784-3788.

Lin, X., J. C. Huang, T. G. Mitchell and J. Heitman (2006). Virulence attributes and hyphal growth of C. neoformans are quantitative traits and the MATalpha allele enhances filamentation. PLoS Genetics 2(11): e187.

Liu, J. Z., S. Jellbauer, A. J. Poe, V. Ton, M. Pesciaroli, T. E. Kehl-Fie, N. A. Restrepo, M. P. Hosking, R. A. Edwards, A. Battistoni, P. Pasquali, T. E. Lane, W. J. Chazin, T. Vogl, J. Roth, E. P. Skaar and M. Raffatellu (2012). Zinc sequestration by the neutrophil protein calprotectin enhances Salmonella growth in the inflamed gut. Cell Host Microbes 11(3): 227-239.

Liu, Z., M. A. Rudd, J. E. Freedman and J. Loscalzo (1998). S-Transnitrosation reactions are involved in the metabolic fate and biological actions of nitric oxide. Journal of Pharmacology and Experimental Therapeutics Ther 284(2): 526-534.

Lledias, F., P. Rangel and W. Hansberg (1998). Oxidation of catalase by singlet oxygen. Journal of Biology and Chemistry273(17): 10630-10637.

Loeb, L. A. and B. D. Preston (1986). Mutagenesis by apurinic/apyrimidinic sites. Annual Review of Genetics 20: 201-230.

Loftin, I. R., S. Franke, S. A. Roberts, A. Weichsel, A. Heroux, W. R. Montfort, C. Rensing and M. M. McEvoy (2005). A novel copper-binding fold for the periplasmic copper resistance protein CusF. Biochemistry 44(31): 10533-10540.

Lu, S., P. B. Killoran, F. C. Fang and L. W. Riley (2002). The global regulator ArcA controls resistance to reactive nitrogen and oxygen intermediates in *Salmonella enterica* serovar Enteritidis. Infection and Immunity 70(2): 451-461.

Lyons, S., L. Wang, J. E. Casanova, S. V. Sitaraman, D. Merlin and A. T. Gewirtz (2004). Salmonella typhimurium transcytoses flagellin via an SPI2-mediated vesicular transport pathway. Journal of Cell Science 117(Pt 24): 5771-5780.

Macomber, L. and J. A. Imlay (2009). The iron-sulfur clusters of dehydratases are primary intracellular targets of copper toxicity. Proceedings of the National Academy of Sciences of the United States of America 106(20): 8344-8349.

Macomber, L., C. Rensing and J. A. Imlay (2007). Intracellular copper does not catalyze the formation of oxidative DNA damage in Escherichia coli. Journal of Bacteriology 189(5): 1616-1626.

Majowicz, S. E., J. Musto, E. Scallan, F. J. Angulo, M. Kirk, S. J. O'Brien, T. F. Jones, A. Fazil and R. M. Hoekstra (2010). The global burden of nontyphoidal Salmonella gastroenteritis. Clinical Infectious Diseases 50(6): 882-889.

Mandal, A. K. and J. M. Arguello (2003). Functional roles of metal binding domains of the Archaeoglobus fulgidus Cu(+)-ATPase CopA. Biochemistry 42(37): 11040-11047.

Masters, S. L., L. A. Mielke, A. L. Cornish, C. E. Sutton, J. O'Donnell, L. H. Cengia, A. W. Roberts, I. P. Wicks, K. H. Mills and B. A. Croker (2010). Regulation of interleukin-1beta by interferon-gamma is species specific, limited by suppressor of cytokine signalling 1 and influences interleukin-17 production. EMBO Reports 11(8): 640-646.

Mastroeni, P., A. Vazquez-Torres, F. C. Fang, Y. Xu, S. Khan, C. E. Hormaeche and G. Dougan (2001). Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. II. Effects on microbial proliferation and host survival *in vivo*. The Journal of Experimental Medicine 192(2): 237-248.

Matsuda, H. and T. Iyanagi (1999). Calmodulin activates intramolecular electron transfer between the two flavins of neuronal nitric oxide synthase flavin domain. Biochimica et Biophysica Acta 1473(2-3): 345-355.

Matsumura, H., K. Onozuka, Y. Terada, Y. Nakano and M. Nakano (1990). Effect of murine recombinant interferon-gamma in the protection of mice against Salmonella. International Journal of Immunopharmacology 12(1): 49-56.

McClelland, M., K. E. Sanderson, J. Spieth, S. W. Clifton, P. Latreille, L. Courtney, S. Porwollik, J. Ali, M. Dante, F. Du, S. Hou, D. Layman, S. Leonard, C. Nguyen, K.

Scott, A. Holmes, N. Grewal, E. Mulvaney, E. Ryan, H. Sun, L. Florea, W. Miller, T. Stoneking, M. Nhan, R. Waterston and R. K. Wilson (2001). Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. Nature 413(6858): 852-856.

McCormick, B. A., C. A. Parkos, S. P. Colgan, D. K. Carnes and J. L. Madara (1998). Apical secretion of a pathogen-elicited epithelial chemoattractant activity in response to surface colonization of intestinal epithelia by Salmonella typhimurium. The Journal of Immunology 160(1): 455-466.

McEwan, A. G., K. Y. Djoko, N. H. Chen, R. L. Counago, S. P. Kidd, A. J. Potter and M. P. Jennings (2011). Novel bacterial MerR-like regulators their role in the response to carbonyl and nitrosative stress. Advances in Microbial Physiology 58: 1-22.

McGhie, E. J., R. D. Hayward and V. Koronakis (2001). Cooperation between actin-binding proteins of invasive Salmonella: SipA potentiates SipC nucleation and bundling of actin. EMBO Journal 20(9): 2131-2139.

McLaggan, D., T. M. Logan, D. G. Lynn and W. Epstein (1990). Involvement of gamma-glutamyl peptides in osmoadaptation of Escherichia coli. Journal of Bacteriology 172(7): 3631-3636.

McLean, S., L. A. Bowman and R. K. Poole (2010). Peroxynitrite stress is exacerbated by flavohaemoglobin-derived oxidative stress in Salmonella Typhimurium and is relieved by nitric oxide. Microbiology 156(Pt 12): 3556-3565.

Mello Filho, A. C., M. E. Hoffmann and R. Meneghini (1984). Cell killing and DNA damage by hydrogen peroxide are mediated by intracellular iron. Biochemical Journal 218(1): 273-275.

Merchant, S. and L. Bogorad (1986). Regulation by copper of the expression of plastocyanin and cytochrome c552 in Chlamydomonas reinhardi. Molecular and Cellular Biology 6(2): 462-469.

Merkel, T. J. and S. Stibitz (1995). Identification of a locus required for the regulation of bvg-repressed genes in Bordetella pertussis. Journal of Bacteriology 177(10): 2727-2736.

Mermod, M., D. Magnani, M. Solioz and J. V. Stoyanov (2012). The copper-inducible ComR (YcfQ) repressor regulates expression of ComC (YcfR), which affects copper permeability of the outer membrane of Escherichia coli. Biometals 25(1): 33-43.

Miao, E. A., C. M. Alpuche-Aranda, M. Dors, A. E. Clark, M. W. Bader, S. I. Miller and A. Aderem (2006). Cytoplasmic flagellin activates caspase-1 and secretion of interleukin 1beta via Ipaf. Nature Immunology 7(6): 569-575.

Mills, C. E., S. Sedelnikova, B. Soballe, M. N. Hughes and R. K. Poole (2001). Escherichia coli flavohaemoglobin (Hmp) with equistoichiometric FAD and haem contents has a

low affinity for dioxygen in the absence or presence of nitric oxide. Biochemical Journal 353(Pt 2): 207-213.

Mills, P. C., D. J. Richardson, J. C. Hinton and S. Spiro (2005). Detoxification of nitric oxide by the flavorubredoxin of *Salmonella enterica* serovar Typhimurium. Biochemical Society Trans 33(Pt 1): 198-199.

Mills, P. C., G. Rowley, S. Spiro, J. C. Hinton and D. J. Richardson (2008). A combination of cytochrome c nitrite reductase (NrfA) and flavorubredoxin (NorV) protects *Salmonella enterica* serovar Typhimurium against killing by NO in anoxic environments. Microbiology 154(Pt 4): 1218-1228.

Monack, D. M., B. Raupach, A. E. Hromockyj and S. Falkow (1996). Salmonella typhimurium invasion induces apoptosis in infected macrophages. Proceedings of the National Academy of Sciences of the United States of America 93(18): 9833-9838.

Monsieurs, P., S. De Keersmaecker, W. W. Navarre, M. W. Bader, F. De Smet, M. McClelland, F. C. Fang, B. De Moor, J. Vanderleyden and K. Marchal (2005). Comparison of the PhoPQ regulon in Escherichia coli and Salmonella typhimurium. Journal of Molecular Evolution 60(4): 462-474.

Morgan, R. W., M. F. Christman, F. S. Jacobson, G. Storz and B. N. Ames (1986). Hydrogen peroxide-inducible proteins in Salmonella typhimurium overlap with heat shock and other stress proteins. Proceedings of the National Academy of Sciences of the United States of America 83(21): 8059-8063.

Müller, J. M., H. W. L. Ziegler-Heitbrock and P. A. Baeuerle (1993). Nuclear factor kappa B, a mediator of lipopolysaccharide effects. Immunobiology 187(3–5): 233-256.

Munson, G. P., D. L. Lam, F. W. Outten and T. V. O'Halloran (2000). Identification of a copper-responsive two-component system on the chromosome of Escherichia coli K-12. Journal of Bacteriology 182(20): 5864-5871.

Murad, F. (1986). Cyclic Guanosine-Monophosphate as a Mediator of Vasodilation. Journal of Clinical Investigation 78(1): 1-5.

Murata, T., W. Tseng, T. Guina, S. I. Miller and H. Nikaido (2007). PhoPQ-mediated regulation produces a more robust permeability barrier in the outer membrane of *Salmonella enterica* serovar typhimurium. Journal of Bacteriology 189(20): 7213-7222.

Nagel de Zwaig, R. and S. E. Luria (1967). Genetics and physiology of colicin-tolerant mutants of Escherichia coli. Journal of Bacteriology 94(4): 1112-1123.

Nauciel, C. and F. Espinasse-Maes (1992). Role of gamma interferon and tumor necrosis factor alpha in resistance to Salmonella typhimurium infection. Infection and Immunity 60(2): 450-454.

Newton, G. L., K. Arnold, M. S. Price, C. Sherrill, S. B. Delcardayre, Y. Aharonowitz, G. Cohen, J. Davies, R. C. Fahey and C. Davis (1996). Distribution of thiols in microorganisms: mycothiol is a major thiol in most actinomycetes. Journal of Bacteriology 178(7): 1990-1995.

Nies, D., M. Mergeay, B. Friedrich and H. G. Schlegel (1987). Cloning of plasmid genes encoding resistance to cadmium, zinc, and cobalt in Alcaligenes eutrophus CH34. Journal of Bacteriology 169(10): 4865-4868.

Nies, D. H. (2003). Efflux-mediated heavy metal resistance in prokaryotes. FEMS Microbiology Reviews 27(2–3): 313-339.

Nishino, K., T. Latifi and E. A. Groisman (2006). Virulence and drug resistance roles of multidrug efflux systems of *Salmonella enterica* serovar Typhimurium. Molecular Microbiology 59(1): 126-141.

Nishino, K., E. Nikaido and A. Yamaguchi (2007). Regulation of multidrug efflux systems involved in multidrug and metal resistance of *Salmonella enterica* serovar Typhimurium. Journal of Bacteriology 189(24): 9066-9075.

Nishioka, H. (1975). Mutagenic activities of metal compounds in bacteria. Mutation Research 31(3): 185-189.

Nose, Y., E. M. Rees and D. J. Thiele (2006). Structure of the Ctr1 copper trans'PORE'ter reveals novel architecture. Trends in Biochemical Sciences 31(11): 604-607.

Nucifora, G., L. Chu, T. K. Misra and S. Silver (1989). Cadmium resistance from Staphylococcus aureus plasmid pI258 cadA gene results from a cadmium-efflux ATPase. Proceedings of the National Academy of Sciences of the United States of America 86(10): 3544-3548.

O'Brien, A. D., D. L. Rosenstreich, I. Scher, G. H. Campbell, R. P. MacDermott and S. B. Formal (1980). Genetic control of susceptibility to Salmonella typhimurium in mice: role of the LPS gene. The Journal of Immunology 124(1): 20-24.

O'Brien, A. D., D. A. Weinstein, M. Y. Soliman and D. L. Rosenstreich (1985). Additional evidence that the Lps gene locus regulates natural resistance to Salmonella Typhimurium in mice. The Journal of Immunology 134(5): 2820-2823.

O'Halloran, T. V. and V. C. Culotta (2000). Metallochaperones, an intracellular shuttle service for metal ions. Journal of Biology and Chemistry275(33): 25057-25060.

Ochman, H. and E. A. Groisman (1996). Distribution of pathogenicity islands in Salmonella spp. Infection and Immunity 64(12): 5410-5412.

Odermatt, A., H. Suter, R. Krapf and M. Solioz (1992). An ATPase operon involved in copper resistance by Enterococcus hirae. Annals of the New York Academy of Sciences 671: 484-486.

Odermatt, A., H. Suter, R. Krapf and M. Solioz (1993). Primary structure of two P-type ATPases involved in copper homeostasis in Enterococcus hirae. Journal of Biology and Chemistry268(17): 12775-12779.

Orskov (1928). Studien über den Infektionsmechanismus bei verschiedenen Paratyphus-Infektionen in weißen Mäusen Zeitschrift für Immunitätsforschung 59: 357–405.

Ortega-Benito, J. M. and P. Langridge (1992). Outbreak of food poisoning due to Salmonella typhimurium DT4 in mayonnaise. Public Health 106(3): 203-208.

Osman, D. and J. S. Cavet (2010). Metal sensing in Salmonella: implications for pathogenesis. Advances in Microbial Physiology 58: 175-232.

Osman, D., C. J. Patterson, K. Bailey, K. Fisher, N. J. Robinson, S. E. Rigby and J. S. Cavet (2013). The copper supply pathway to a Salmonella Cu,Zn-superoxide dismutase (SodCII) involves P(1B)-type ATPase copper efflux and periplasmic CueP. Molecular Microbiology 87(3): 466-477.

Outten, C. E. and T. V. O'Halloran (2001). Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis. Science 292(5526): 2488-2492.

Outten, F. W., D. L. Huffman, J. A. Hale and T. V. O'Halloran (2001). The independent cue and cus systems confer copper tolerance during aerobic and anaerobic growth in Escherichia coli. Journal of Biology and Chemistry 276(33): 30670-30677.

Outten, F. W., C. E. Outten, J. Hale and T. V. O'Halloran (2000). Transcriptional activation of an Escherichia coli copper efflux regulon by the chromosomal MerR homologue, cueR. Journal of Biology and Chemistry 275(40): 31024-31029.

Palumaa, P., L. Kangur, A. Voronova and R. Sillard (2004). Metal-binding mechanism of Cox17, a copper chaperone for cytochrome c oxidase. Biochemical Journal 382: 307-314.

Park, B. Y. and J. Chung (2008). Effects of various metal ions on the gene expression of iron exporter ferroportin-1 in J774 macrophages. Nutrition Research and Practice 2(4): 317-321.

Parkhill, J., G. Dougan, K. D. James, N. R. Thomson, D. Pickard, J. Wain, C. Churcher, K. L. Mungall, S. D. Bentley, M. T. G. Holden, M. Sebaihia, S. Baker, D. Basham, K. Brooks, T. Chillingworth, P. Connerton, A. Cronin, P. Davis, R. M. Davies, L. Dowd, N. White, J. Farrar, T. Feltwell, N. Hamlin, A. Haque, T. T. Hien, S. Holroyd, K. Jagels, A. Krogh, T. S. Larsen, S. Leather, S. Moule, P. O'Gaora, C. Parry, M. Quail, K. Rutherford, M. Simmonds, J. Skelton, K. Stevens, S. Whitehead and B. G. Barrell (2001). Complete genome sequence of a multiple drug resistant *Salmonella enterica* serovar Typhi CT18. Nature 413(6858): 848-852.

Parvatiyar, K., E. M. Alsabbagh, U. A. Ochsner, M. A. Stegemeyer, A. G. Smulian, S. H. Hwang, C. R. Jackson, T. R. McDermott and D. J. Hassett (2005). Global analysis of cellular factors and responses involved in Pseudomonas aeruginosa resistance to arsenite. Journal of Bacteriology 187(14): 4853-4864.

Paulsen, I. T., J. H. Park, P. S. Choi and M. H. Saier, Jr. (1997). A family of gram-negative bacterial outer membrane factors that function in the export of proteins, carbohydrates, drugs and heavy metals from gram-negative bacteria. FEMS Microbiol Letters 156(1): 1-8.

Pearce, L. L., K. Wasserloos, C. M. St Croix, R. Gandley, E. S. Levitan and B. R. Pitt (2000). Metallothionein, nitric oxide and zinc homeostasis in vascular endothelial cells. Journal of Nutrition 130(5S Suppl): 1467S-1470S.

Pedersen, P. L. and E. Carafoli (1987). Ion motive ATPases. I. Ubiquity, properties, and significance to cell function. Trends in Biochemical Sciences 12: 146-150.

Pereira, Y., G. Lagniel, E. Godat, P. Baudouin-Cornu, C. Junot and J. Labarre (2008). Chromate causes sulfur starvation in yeast. Toxicological Sciences 106(2): 400-412.

Petris, M. J., J. F. B. Mercer, J. G. Culvenor, P. Lockhart, P. A. Gleeson and J. Camakaris (1996). Ligand-regulated transport of the Menkes copper P-type ATPase efflux pump from the Golgi apparatus to the plasma membrane: A novel mechanism of regulated trafficking. Embo Journal 15(22): 6084-6095.

Petris, M. J., K. Smith, J. Lee and D. J. Thiele (2003). Copper-stimulated endocytosis and degradation of the human copper transporter, hCtr1. Journal of Biology and Chemistry 278(11): 9639-9646.

Plant, J. E., J. M. Blackwell, A. D. O'Brien, D. J. Bradley and A. A. Glynn (1982). Are the Lsh and Ity disease resistance genes at one locus on mouse chromosome 1? Nature 297(5866): 510-511.

Poltorak, A., X. He, I. Smirnova, M. Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Ricciardi-Castagnoli, B. Layton and B. Beutler (1998). Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 282(5396): 2085-2088.

Pontel, L. B., M. E. Audero, M. Espariz, S. K. Checa and F. C. Soncini (2007). GolS controls the response to gold by the hierarchical induction of Salmonella-specific genes that include a CBA efflux-coding operon. Molecular Microbiology 66(3): 814-825.

Pontel, L. B., A. Pezza and F. C. Soncini (2010). Copper stress targets the rcs system to induce multiaggregative behavior in a copper-sensitive Salmonella strain. Journal of Bacteriology 192(23): 6287-6290.

Poole, K., D. E. Heinrichs and S. Neshat (1993). Cloning and sequence analysis of an EnvCD homologue in Pseudomonas aeruginosa: regulation by iron and possible

involvement in the secretion of the siderophore pyoverdine. Molecular Microbiology 10(3): 529-544.

Portmann, R., D. Magnani, J. V. Stoyanov, A. Schmechel, G. Multhaup and M. Solioz (2004). Interaction kinetics of the copper-responsive CopY repressor with the cop promoter of Enterococcus hirae. Journal of Biological Inorganic Chemistry 9(4): 396-402.

Possel, H., H. Noack, J. Putzke, G. Wolf and H. Sies (2000). Selective upregulation of inducible nitric oxide synthase (iNOS) by lipopolysaccharide (LPS) and cytokines in microglia: in vitro and *in vivo* studies. Glia 32(1): 51-59.

Prior, K., I. Hautefort, J. C. Hinton, D. J. Richardson and G. Rowley (2009). All stressed out. Salmonella pathogenesis and reactive nitrogen species. Advances in Microbial Physiology 56: 1-28.

Pullamsetti, S. S., D. Maring, H. A. Ghofrani, K. Mayer, N. Weissmann, B. Rosengarten, M. Lehner, C. Schudt, R. Boer, F. Grimminger, W. Seeger and R. T. Schermuly (2006). Effect of nitric oxide synthase (NOS) inhibition on macro- and microcirculation in a model of rat endotoxic shock. Thromb Haemost 95(4): 720-727.

Rae, T. D., P. J. Schmidt, R. A. Pufahl, V. C. Culotta and T. V. O'Halloran (1999). Undetectable intracellular free copper: the requirement of a copper chaperone for superoxide dismutase. Science 284(5415): 805-808.

Raimunda, D., M. Gonzalez-Guerrero, B. W. Leeber, 3rd and J. M. Arguello (2011). The transport mechanism of bacterial Cu+-ATPases: distinct efflux rates adapted to different function. Biometals 24(3): 467-475.

Ramarathinam, L., R. A. Shaban, D. W. Niesel and G. R. Klimpel (1991). Interferon gamma (IFN-gamma) production by gut-associated lymphoid tissue and spleen following oral Salmonella typhimurium challenge. Microbial Pathogenesis 11(5): 347-356.

Rees, E. M. and D. J. Thiele (2007). Identification of a vacuole-associated metalloreductase and its role in Ctr2-mediated intracellular copper mobilization. Journal of Biology and Chemistry282(30): 21629-21638.

Rensing, C., B. Fan, R. Sharma, B. Mitra and B. P. Rosen (2000). CopA: An Escherichia coli Cu(I)-translocating P-type ATPase. Proceedings of the National Academy of Sciences of the United States of America 97(2): 652-656.

Ricci, V. and L. J. Piddock (2010). Exploiting the role of TolC in pathogenicity: identification of a bacteriophage for eradication of Salmonella serovars from poultry. Applied and Environmental Microbiology 76(5): 1704-1706.

Richardson, A. R., E. C. Payne, N. Younger, J. E. Karlinsey, V. C. Thomas, L. A. Becker, W. W. Navarre, M. E. Castor, S. J. Libby and F. C. Fang (2011). Multiple targets of nitric

oxide in the tricarboxylic acid cycle of *Salmonella enterica* serovar typhimurium. Cell Host Microbe 10(1): 33-43.

Richter, C. (1987). Biophysical consequences of lipid peroxidation in membranes. Chemistry and Physics of Lipids 44(2-4): 175-189.

Ridge, P. G., Y. Zhang and V. N. Gladyshev (2008). Comparative genomic analyses of copper transporters and cuproproteomes reveal evolutionary dynamics of copper utilization and its link to oxygen. PLoS One 3(1): e1378.

Roberts, S. A., A. Weichsel, G. Grass, K. Thakali, J. T. Hazzard, G. Tollin, C. Rensing and W. R. Montfort (2002). Crystal structure and electron transfer kinetics of CueO, a multicopper oxidase required for copper homeostasis in Escherichia coli. Proceedings of the National Academy of Sciences of the United States of America 99(5): 2766-2771.

Robinson, N. J. and D. R. Winge (2010). Copper Metallochaperones. Annual Review of Biochemistry, Vol 79 79: 537-562.

Rock, J. D., M. J. Thomson, R. C. Read and J. W. Moir (2007). Regulation of denitrification genes in Neisseria meningitidis by nitric oxide and the repressor NsrR. Journal of Bacteriology 189(3): 1138-1144.

Rodriguez-Granillo, A., A. Crespo and P. Wittung-Stafshede (2010). Interdomain interactions modulate collective dynamics of the metal-binding domains in the Wilson disease protein. The Journal of Physical Chemistry B 114(5): 1836-1848.

Romero, N., A. Denicola, J. M. Souza and R. Radi (1999). Diffusion of peroxynitrite in the presence of carbon dioxide. Archives of Biochemistry and Biophysics 368(1): 23-30.

Rose, F., M. Hodak and J. Bernholc (2011). Mechanism of copper(II)-induced misfolding of Parkinson's disease protein. Scientific Reports 1: 11.

Rosenberger, C. M. and B. B. Finlay (2003). Phagocyte sabotage: disruption of macrophage signalling by bacterial pathogens. Nat Rev Molecular and Cellular Biology 4(5): 385-396.

Rosenzweig, A. C. (2002). Metallochaperones: Bind and deliver. Chemistry & Biology 9(6): 673-677.

Rosner, J. L. and R. G. Martin (2009). An excretory function for the Escherichia coli outer membrane pore TolC: upregulation of marA and soxS transcription and Rob activity due to metabolites accumulated in tolC mutants. Journal of Bacteriology 191(16): 5283-5292.

Ruby, E. G., M. Urbanowski, J. Campbell, A. Dunn, M. Faini, R. Gunsalus, P. Lostroh, C. Lupp, J. McCann, D. Millikan, A. Schaefer, E. Stabb, A. Stevens, K. Visick, C. Whistler and E. P. Greenberg (2005). Complete genome sequence of Vibrio fischeri:

a symbiotic bacterium with pathogenic congeners. Proceedings of the National Academy of Sciences of the United States of America 102(8): 3004-3009.

Rudolph, M. G., C. Weise, S. Mirold, B. Hillenbrand, B. Bader, A. Wittinghofer and W. D. Hardt (1999). Biochemical analysis of SopE from Salmonella typhimurium, a highly efficient guanosine nucleotide exchange factor for RhoGTPases. Journal of Biology and Chemistry274 (43): 30501-30509.

Rychlik, I. and P. A. Barrow (2005). Salmonella stress management and its relevance to behaviour during intestinal colonisation and infection. FEMS Microbiology Reviews 29(5): 1021-1040.

Santos, M. R., A. M. Cosme, J. D. Becker, J. M. Medeiros, M. F. Mata and L. M. Moreira (2010). Absence of functional TolC protein causes increased stress response gene expression in Sinorhizobium meliloti. BMC Microbiology 10: 180.

Santos, R. L., S. Zhang, R. M. Tsolis, R. A. Kingsley, L. G. Adams and A. J. Baumler (2001). Animal models of Salmonella infections: enteritis versus typhoid fever. Microbes and Infection 3(14-15): 1335-1344.

Sarih, M., V. Souvannavong and A. Adam (1993). Nitric Oxide Synthase Induces Macrophage Death by Apoptosis. Biochemical and Biophysical Research Communications 191(2): 503-508.

Saura, M., C. Zaragoza, C. Bao, A. McMillan and C. J. Lowenstein (1999). Interaction of interferon regulatory factor-1 and nuclear factor kappaB during activation of inducible nitric oxide synthase transcription. Journal of Molecular Biology 289(3): 459-471.

Schapiro, J. M., S. J. Libby and F. C. Fang (2003). Inhibition of bacterial DNA replication by zinc mobilization during nitrosative stress. Proceedings of the National Academy of Sciences of the United States of America 100(14): 8496-8501.

Schelder, S., D. Zaade, B. Litsanov, M. Bott and M. Brocker (2011). The two-component signal transduction system CopRS of Corynebacterium glutamicum is required for adaptation to copper-excess stress. PLoS One 6(7): e22143.

Schmidt, T. and H. G. Schlegel (1994). Combined nickel-cobalt-cadmium resistance encoded by the ncc locus of Alcaligenes xylosoxidans 31A. Journal of Bacteriology 176(22): 7045-7054.

Schwan, W. R., P. Warrener, E. Keunz, C. K. Stover and K. R. Folger (2005). Mutations in the cueA gene encoding a copper homeostasis P-type ATPase reduce the pathogenicity of Pseudomonas aeruginosa in mice. International Journal of Medical Microbiology 295(4): 237-242.

Sharan, R., S. Chhibber and R. H. Reed (2011). A murine model to study the antibacterial effect of copper on infectivity of *Salmonella enterica* serovar Typhimurium. International Journal of Environmental Research and Public Health 8(1): 21-36.

Sharrocks, A. D., J. Green and J. R. Guest (1990). *In vivo* and in vitro mutants of FNR the anaerobic transcriptional regulator of E. coli. FEBS Letters 270(1-2): 119-122.

Shea, J. E., M. Hensel, C. Gleeson and D. W. Holden (1996). Identification of a virulence locus encoding a second type III secretion system in Salmonella typhimurium. Proceedings of the National Academy of Sciences of the United States of America 93(6): 2593-2597.

Shelobolina, E. S., S. A. Sullivan, K. R. O'Neill, K. P. Nevin and D. R. Lovley (2004). Isolation, characterization, and U(VI)-reducing potential of a facultatively anaerobic, acid-resistant Bacterium from Low-pH, nitrate- and U(VI)-contaminated subsurface sediment and description of *Salmonella subterranea* sp. nov. Applied and Environmental Microbiology 70(5): 2959-2965.

Sheppard, M., C. Webb, F. Heath, V. Mallows, R. Emilianus, D. Maskell and P. Mastroeni (2003). Dynamics of bacterial growth and distribution within the liver during Salmonella infection. Cell Microbiology 5(9): 593-600.

Sheth, A. N., M. Hoekstra, N. Patel, G. Ewald, C. Lord, C. Clarke, E. Villamil, K. Niksich, C. Bopp, T. A. Nguyen, D. Zink and M. Lynch (2011). A national outbreak of Salmonella serotype Tennessee infections from contaminated peanut butter: a new food vehicle for salmonellosis in the United States. Clinical Infectious Diseases 53(4): 356-362.

Singh, R. J., N. Hogg, J. Joseph and B. Kalyanaraman (1996). Mechanism of nitric oxide release from S-nitrosothiols. Journal of Biology and Chemistry271(31): 18596-18603.

Solioz, M., A. Odermatt and R. Krapf (1994). Copper pumping ATPases: common concepts in bacteria and man. FEBS Letters 346(1): 44-47.

Solomon, E. I., U. M. Sundaram and T. E. Machonkin (1996). Multicopper Oxidases and Oxygenases. Chemical Reviews 96(7): 2563-2606.

Soncini, F. C., E. Garcia Vescovi, F. Solomon and E. A. Groisman (1996). Molecular basis of the magnesium deprivation response in Salmonella typhimurium: identification of PhoP-regulated genes. Journal of Bacteriology 178(17): 5092-5099.

Song, M., M. Husain, J. Jones-Carson, L. Liu, C. A. Henard and A. Vazquez-Torres (2013). Low-molecular-weight thiol-dependent antioxidant and antinitrosative defences in Salmonella pathogenesis. Molecular Microbiology 87(3): 609-622.

Sperber, S. J. and C. J. Schleupner (1987). Salmonellosis during infection with human immunodeficiency virus. Rev Infect Dis 9(5): 925-934.

Stamler, J. S., D. J. Singel and J. Loscalzo (1992). Biochemistry of nitric oxide and its redox-activated forms. Science 258(5090): 1898-1902.

Stein, M. A., K. Y. Leung, M. Zwick, F. Garcia-del Portillo and B. B. Finlay (1996). Identification of a Salmonella virulence gene required for formation of filamentous

structures containing lysosomal membrane glycoproteins within epithelial cells. Molecular Microbiology 20(1): 151-164.

Stender, S., A. Friebel, S. Linder, M. Rohde, S. Mirold and W. D. Hardt (2000). Identification of SopE2 from Salmonella typhimurium, a conserved guanine nucleotide exchange factor for Cdc42 of the host cell. Molecular Microbiology 36(6): 1206-1221.

Stevanin, T. M., N. Ioannidis, C. E. Mills, S. O. Kim, M. N. Hughes and R. K. Poole (2000). Flavohemoglobin Hmp affords inducible protection for Escherichia coli respiration, catalyzed by cytochromes bo' or bd, from nitric oxide. Journal of Biology and Chemistry 275(46): 35868-35875.

Stevanin, T. M., R. K. Poole, E. A. Demoncheaux and R. C. Read (2002). Flavohemoglobin Hmp protects *Salmonella enterica* serovar typhimurium from nitric oxide-related killing by human macrophages. Infection and Immunity 70(8): 4399-4405.

Stevanin, T. M., R. C. Read and R. K. Poole (2007). The hmp gene encoding the NO-inducible flavohaemoglobin in Escherichia coli confers a protective advantage in resisting killing within macrophages, but not in vitro: links with swarming motility. Gene 398(1-2): 62-68.

Stewart, C. R., D. M. Burnside and N. P. Cianciotto (2011). The surfactant of Legionella pneumophila Is secreted in a TolC-dependent manner and is antagonistic toward other Legionella species. Journal of Bacteriology 193(21): 5971-5984.

Stock, A. M., V. L. Robinson and P. N. Goudreau (2000). Two-component signal transduction. Annual Review of Biochemistry 69: 183-215.

Stoyanov J.V., and Brown N. J. (2003) The *Escherichia coli* copper-responsive *copA* promoter is activated by gold. Journal of Biological Chemistry 287(3): 1407-1410.

Strauch, K. L., J. B. Lenk, B. L. Gamble and C. G. Miller (1985). Oxygen regulation in Salmonella typhimurium. Journal of Bacteriology 161(2): 673-680.

Stuehr, D. J. (1999). Mammalian nitric oxide synthases.

Szabo, C. and H. Ohshima (1997). DNA damage induced by peroxynitrite: subsequent biological effects. Nitric Oxide 1(5): 373-385.

Tak, P. P. and G. S. Firestein (2001). NF-kappaB: a key role in inflammatory diseases. Journal of Clinical Investigation 107(1): 7-11.

Takeuchi, A. and H. Sprinz (1967). Electron-microscope studies of experimental salmonella infection in the preconditioned guinea pig: II. response of the intestinal mucosa to the invasion by Salmonella Typhimurium. American Journal of Pathology 51(1): 137-161.

Talbot, S., S. Totemeyer, M. Yamamoto, S. Akira, K. Hughes, D. Gray, T. Barr, P. Mastroeni, D. J. Maskell and C. E. Bryant (2009). Toll-like receptor 4 signalling through MyD88

is essential to control *Salmonella enterica* serovar typhimurium infection, but not for the initiation of bacterial clearance. Immunology 128(4): 472-483.

Tanzi, R. E., K. Petrukhin, I. Chernov, J. L. Pellequer, W. Wasco, B. Ross, D. M. Romano, E. Parano, L. Pavone, L. M. Brzustowicz and et al. (1993). The Wilson disease gene is a copper transporting ATPase with homology to the Menkes disease gene. Nature Genetics 5(4): 344-350.

Taylor, C. M., D. Osman and J. S. Cavet (2009). Differential expression from two iron-responsive promoters in *Salmonella enterica* serovar Typhimurium reveals the presence of iron in macrophage-phagosomes. Microbial Pathogenesis 46(2): 114-118.

Tennant, S. M., E. L. Hartland, T. Phumoonna, D. Lyras, J. I. Rood, R. M. Robins-Browne and I. R. van Driel (2008). Influence of gastric acid on susceptibility to infection with ingested bacterial pathogens. Infection and Immunity 76(2): 639-645.

Tetaz (1983). Plasmid-controlled resistance to copper in Escherichia coli. Journal of Bacteriology 154(4): 1263-1268.

Thanabalu, T., E. Koronakis, C. Hughes and V. Koronakis (1998). Substrate-induced assembly of a contiguous channel for protein export from E.coli: reversible bridging of an inner-membrane translocase to an outer membrane exit pore. EMBO Journal 17(22): 6487-6496.

Thiennimitr, P., S. E. Winter, M. G. Winter, M. N. Xavier, V. Tolstikov, D. L. Huseby, T. Sterzenbach, R. M. Tsolis, J. R. Roth and A. J. Baumler (2011). Intestinal inflammation allows Salmonella to use ethanolamine to compete with the microbiota. Proceedings of the National Academy of Sciences of the United States of America 108(42): 17480-17485.

Torres, J., V. Darley-Usmar and M. T. Wilson (1995). Inhibition of cytochrome c oxidase in turnover by nitric oxide: mechanism and implications for control of respiration. Biochemical Journal 312: 169-173.

Tottey, S., C. J. Patterson, L. Banci, I. Bertini, I. C. Felli, A. Pavelkova, S. J. Dainty, R. Pernil, K. J. Waldron, A. W. Foster and N. J. Robinson (2012). Cyanobacterial metallochaperone inhibits deleterious side reactions of copper. Proceedings of the National Academy of Sciences of the United States of America 109(1): 95-100.

Tree, J. J., S. P. Kidd, M. P. Jennings and A. G. McEwan (2005). Copper sensitivity of cueO mutants of Escherichia coli K-12 and the biochemical suppression of this phenotype. Biochemical and Biophysical Research Communications 328(4): 1205-1210.

Tsai, B. and T. A. Rapoport (2002). Unfolded cholera toxin is transferred to the ER membrane and released from protein disulfide isomerase upon oxidation by Ero1. The Journal of Cell Biology 159(2): 207-216.

Tseng, T. T., K. S. Gratwick, J. Kollman, D. Park, D. H. Nies, A. Goffeau and M. H. Saier, Jr. (1999). The RND permease superfamily: an ancient, ubiquitous and diverse family that includes human disease and development proteins. Journal of Molecular Microbiology and Biotechnology 1(1): 107-125.

Tucker, N. P., M. G. Hicks, T. A. Clarke, J. C. Crack, G. Chandra, N. E. Le Brun, R. Dixon and M. I. Hutchings (2008). The transcriptional repressor protein NsrR senses nitric oxide directly via a [2Fe-2S] cluster. PLoS One 3(11): e3623.

Tumer, Z. and L. B. Moller (2010). Menkes disease. Eur J Hum Genet 18(5): 511-518.

Uchiya, K., M. A. Barbieri, K. Funato, A. H. Shah, P. D. Stahl and E. A. Groisman (1999). A Salmonella virulence protein that inhibits cellular trafficking. EMBO Journal 18(14): 3924-3933.

Umezawa, K., T. Akaike, S. Fujii, M. Suga, K. Setoguchi, A. Ozawa and H. Maeda (1997). Induction of nitric oxide synthesis and xanthine oxidase and their roles in the antimicrobial mechanism against Salmonella typhimurium infection in mice. Infection and Immunity 65(7): 2932-2940.

Uzzau, S., L. Bossi and N. Figueroa-Bossi (2002). Differential accumulation of Salmonella[Cu, Zn] superoxide dismutases SodCI and SodCII in intracellular bacteria: correlation with their relative contribution to pathogenicity. Molecular Microbiology 46(1): 147-156.

Vakharia, H., G. J. German and R. Misra (2001). Isolation and characterization of Escherichia coli tolC mutants defective in secreting enzymatically active alpha-hemolysin. Journal of Bacteriology 183(23): 6908-6916.

van Wonderen, J. H., B. Burlat, D. J. Richardson, M. R. Cheesman and J. N. Butt (2008). The nitric oxide reductase activity of cytochrome c nitrite reductase from Escherichia coli. Journal of Biology and Chemistry 283(15): 9587-9594.

Vanin, A. F., I. V. Malenkova and V. A. Serezhenkov (1997). Iron catalyzes both decomposition and synthesis of S-nitrosothiols: optical and electron paramagnetic resonance studies. Nitric Oxide 1(3): 191-203.

Varesio, L., E. Blasi, G. B. Thurman, J. E. Talmadge, R. H. Wiltrout and R. B. Herberman (1984). Potent activation of mouse macrophages by recombinant interferon-gamma. Cancer Research 44(10): 4465-4469.

Vasudevan, S. G., W. L. Armarego, D. C. Shaw, P. E. Lilley, N. E. Dixon and R. K. Poole (1991). Isolation and nucleotide sequence of the hmp gene that encodes a haemoglobin-like protein in Escherichia coli K-12. Molecular Genetics and Genomics 226(1-2): 49-58.

Vazquez-Torres, A., J. Jones-Carson, A. J. Baumler, S. Falkow, R. Valdivia, W. Brown, M. Le, R. Berggren, W. T. Parks and F. C. Fang (1999). Extraintestinal dissemination of Salmonella by CD18-expressing phagocytes. Nature 401(6755): 804-808.

Vazquez-Torres, A., J. Jones-Carson, P. Mastroeni, H. Ischiropoulos and F. C. Fang (2000). Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. I. Effects on microbial killing by activated peritoneal macrophages in vitro. The Journal of Experimental Medicine 192(2): 227-236.

Vazquez-Torres, A., Y. Xu, J. Jones-Carson, D. W. Holden, S. M. Lucia, M. C. Dinauer, P. Mastroeni and F. C. Fang (2000). Salmonella pathogenicity island 2-dependent evasion of the phagocyte NADPH oxidase. Science 287(5458): 1655-1658.

Veeravalli, K., D. Boyd, B. L. Iverson, J. Beckwith and G. Georgiou (2011). Laboratory evolution of glutathione biosynthesis reveals natural compensatory pathways. Nature Chemical Biology 7(2): 101-105.

Vulpe, C., B. Levinson, S. Whitney, S. Packman and J. Gitschier (1993). Isolation of a candidate gene for Menkes disease and evidence that it encodes a copper-transporting ATPase. Nature Genetics 3(1): 7-13.

Warner, D. M. and S. B. Levy (2010). Different effects of transcriptional regulators MarA, SoxS and Rob on susceptibility of Escherichia coli to cationic antimicrobial peptides (CAMPs): Rob-dependent CAMP induction of the marRAB operon. Microbiology 156(Pt 2): 570-578.

Webber, M. A., A. M. Bailey, J. M. Blair, E. Morgan, M. P. Stevens, J. C. Hinton, A. Ivens, J. Wain and L. J. Piddock (2009). The global consequence of disruption of the AcrAB-TolC efflux pump in *Salmonella enterica* includes reduced expression of SPI-1 and other attributes required to infect the host. Journal of Bacteriology 191(13): 4276-4285.

Wefers, H., D. Schulte-Frohlinde and H. Sies (1987). Loss of transforming activity of plasmid DNA (pBR322) in E. coli caused by singlet molecular oxygen. FEBS Letters 211(1): 49-52.

Weiss, J., M. Victor, O. Stendhal and P. Elsbach (1982). Killing of gram-negative bacteria by polymorphonuclear leukocytes: role of an O_2^- independent bactericidal system. Journal of Clinical Investigation 69(4): 959-970.

Wenk, G. and K. Suzuki (1983). Congenital copper deficiency: copper therapy and dopamine-beta-hydroxylase activity in the mottled (brindled) mouse. Journal of Neurochemistry 41(6): 1648-1652.

White (1926). Medical Research Council. Further studies of the Salmonella Group. Analyst 51(609): 632-633.

White, C., J. Lee, T. Kambe, K. Fritsche and M. J. Petris (2009). A role for the ATP7A copper-transporting ATPase in macrophage bactericidal activity. Journal of Biology and Chemistry284(49): 33949-33956.

Williams, D. L. H. (1999). The chemistry of S-nitrosothiols. Accounts of Chemical Research 32(10): 869-876.

Williams, M. J., A. Rodriguez, D. A. Kimbrell and E. D. Eldon (1997). The 18-wheeler mutation reveals complex antibacterial gene regulation in Drosophila host defense. EMBO Journal 16(20): 6120-6130.

Winter, S. E., P. Thiennimitr, M. G. Winter, B. P. Butler, D. L. Huseby, R. W. Crawford, J. M. Russell, C. L. Bevins, L. G. Adams, R. M. Tsolis, J. R. Roth and A. J. Baumler (2010). Gut inflammation provides a respiratory electron acceptor for Salmonella. Nature 467(7314): 426-429.

Wolschendorf, F., D. Ackart, T. B. Shrestha, L. Hascall-Dove, S. Nolan, G. Lamichhane, Y. Wang, S. H. Bossmann, R. J. Basaraba and M. Niederweis (2011). Copper resistance is essential for virulence of Mycobacterium tuberculosis. Proceedings of the National Academy of Sciences of the United States of America 108(4): 1621-1626.

Wong, C. M., Y. Zhou, R. W. Ng, H. F. Kung Hf and D. Y. Jin (2002). Cooperation of yeast peroxiredoxins Tsa1p and Tsa2p in the cellular defense against oxidative and nitrosative stress. Journal of Biology and Chemistry 277(7): 5385-5394.

Woolum, J. C., E. Tiezzi and B. Commoner (1968). Electron spin resonane of iron-nitric oxide complexes with amino acids, peptides and proteins. Biochimica et Biophysica Acta 160(3): 311-320.

Wray, C. and W. J. Sojka (1978). Experimental Salmonella typhimurium infection in calves. Research in Veterinary Science 25(2): 139-143.

Wurfel, M. M., S. T. Kunitake, H. Lichenstein, J. P. Kane and S. D. Wright (1994). Lipopolysaccharide (LPS)-binding protein is carried on lipoproteins and acts as a cofactor in the neutralization of LPS. The Journal of Experimental Medicine 180(3): 1025-1035.

Yaganza, E. S., D. Rioux, M. Simard, J. Arul and R. J. Tweddell (2004). Ultrastructural alterations of Erwinia carotovora subsp. atroseptica caused by treatment with aluminum chloride and sodium metabisulfite. Applied and Environmental Microbiology 70(11): 6800-6808.

Yu, Y., H. Zeng, S. Lyons, A. Carlson, D. Merlin, A. S. Neish and A. T. Gewirtz (2003). TLR5-mediated activation of p38 MAPK regulates epithelial IL-8 expression via posttranscriptional mechanism. American Journal of Physiology - Gastrointestinal and Liver Physiology 285(2): G282-290.

Zaragoza, C., C. J. Ocampo, M. Saura, A. McMillan and C. J. Lowenstein (1997). Nitric oxide inhibition of coxsackievirus replication in vitro. Journal of Clinical Investigation 100(7): 1760-1767.

Zhang, S., R. L. Santos, R. M. Tsolis, S. Stender, W. D. Hardt, A. J. Baumler and L. G. Adams (2002). The *Salmonella enterica* serotype typhimurium effector proteins SipA, SopA, SopB, SopD, and SopE2 act in concert to induce diarrhea in calves. Infection and Immunity 70(7): 3843-3855.

Zhou, B. and J. Gitschier (1997). hCTR1: a human gene for copper uptake identified by complementation in yeast. Proceedings of the National Academy of Sciences of the United States of America 94(14): 7481-7486.

Zhou, D., L. M. Chen, L. Hernandez, S. B. Shears and J. E. Galan (2001). A Salmonella inositol polyphosphatase acts in conjunction with other bacterial effectors to promote host cell actin cytoskeleton rearrangements and bacterial internalization. Molecular Microbiology 39(2): 248-259.

Zwilling, B. S., D. E. Kuhn, L. Wikoff, D. Brown and W. Lafuse (1999). Role of iron in Nramp1-mediated inhibition of mycobacterial growth. Infection and Immunity 67(3): 1386-1392.