The Contribution of Nitric Oxide Detoxification and Nitrous Oxide Production to *Salmonella* Pathogenesis

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Abstract

Salmonella is responsible for millions of infections worldwide, with varied disease outcomes, ranging from localised gastroenteritis to a fatal systemic infection. As a facultative anaerobe, *Salmonella* is able to respire using nitrate as an alternative electron acceptor. This study highlights the role of two gaseous intermediates produced during nitrate respiration, nitric oxide (NO) and nitrous oxide (N_2O), during *Salmonella* pathogenesis.

Building from previous studies, a link is drawn between the high level of production of N_2O by *Salmonella*, and the metabolically expensive, and seemingly unimportant synthesis of vitamin B_{12}. We demonstrate the importance of the vitamin B_{12} independent methionine synthase, MetE, both during nitrate respiration and for survival within macrophages. We hypothesise that when respiring on nitrate in the intestine *Salmonella* releases N_2O which is detrimental to vitamin B_{12} availability and commensal survival in the intestine. *Salmonella* however is protected two-fold from the toxicity of N_2O, by the presence of MetE, and by the ability to synthesise vitamin B_{12} *de novo*. This phenomenon is further investigated across multiple *Salmonella* serovars, with the indication that enteric strains produce both higher levels of vitamin B_{12} and N_2O.

As well as during nitrate respiration, *Salmonella* also encounters NO exogenously when challenged by the host defense inside macrophages. Since NO is a highly toxic compound effective detoxification systems are essential for survival. A relationship between NO and tellurite resistance in *Salmonella* is shown. Three putative tellurite resistance proteins, TehB, STM1808 and YeaR were confirmed to provide resistance against tellurite in *Salmonella* and share functional redundancy in the anaerobic detoxification of NO and infection of macrophages. Using a suite of NO sensitive mutants, we also demonstrate a clear correlation between tellurite and NO sensitivity.

Collectively this study highlights two important aspects of the nitrogen cycle which contribute to pathogenesis in *Salmonella*.

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Abbreviations

Amp	Ampillicin
Bp	Base Pair
B_{12}	Vitamin B_{12}
Cm	Chloramphenicol
Cfu	Colony forming units
ºC	Degrees Celsius
dH_2O	Distilled water
DMEM	Dulbecco's modified Eagle medium
DUF	Domain of unknown function
DNA	Deoxyribonucleic acid
FLP	Flip recombinase
g	Grams
IFN-γ	Interferon gamma
iNOS	Inducible nitric oxide synthase
Kb	Kilobase-pair
Kan	Kanamycin
LB	Luria-Bertani
L-NAME	N-Nitroarginine methyl ester
µg	Micrograms
µM	Micromolar

M	Molar
MDR	Multi-drug resistant
mg	Milligrams
ml	Millileters
mM	Millimolar
min	Minutes
MOI	Multiplicity of infection
N_2	Dinitrogen
N_2O	Nitrous oxide
NH_4^+	Ammonium
NO	Nitric oxide
NO_2	Nitrite
NO_3	Nitrate
OD	Optical density
ONOO	Peroxynitrite
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Ppb	Parts per billion
Ppm	Parts per million
RNS	Reactive nitrogen species
ROS	Reactive oxygen species

SD	Standard deviation
SE	Standard error
TeO_3	Tellurite

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1 Introduction

1.1 General

Salmonella is a genus of Gram negative bacteria which contains just two species: *Salmonella bongori* and *Salmonella enterica*. Within *enterica* there are over 2500 different *Salmonella* serovars, a sub-species categorisation system based on outer membrane antigens, and includes all of those which cause disease in humans (Ochman and Groisman 1994).

Only about 50 of these serovars have been isolated as known pathogens (Coburn, Grassl and Finlay 2007). Different serovars of *Salmonella* can infect different hosts, some are host specific, and restricted to a single host, whilst others are generalists and have the ability to infect a wide range of mammalian and avian hosts. There are two main disease types associated with *Salmonella.* A systemic typhoidal infection caused by *S*. Typhi in humans and more common non-typhoidal strains which cause localised gastrointestinal/enteric infections (enteritis), such as *S*. Typhimurium in humans and *S*. Dublin in calves (Coburn et al. 2007)

Salmonella Typhi and Paratyphi (see 1.4.2 Enteric fever) almost exclusively cause a systemic infection in humans and therefore are referred to as host restricted strains. Similarly, *Salmonella* Gallinarum is only known to cause a systemic infection in chickens/fowl. *Salmonella* Cholerasuis and *Salmonella* Dublin are most commonly known to cause a systemic infection in pigs and cattle respectively, but also have the ability to infect other hosts, including humans, these serovars are referred to as host adapted strains, since they most commonly infect a single host but it is not a strict restriction. There are then further strains such as *Salmonella* Typhimurium and *Salmonella* Enteritidis which cause an enteric infection in a wide range of hosts, and as such are un-restricted serovars (Uzzau et al. 2000). The most common non-typhoidal serovar used as a model laboratory strain is *Salmonella enterica* subs. *enterica* serovar Typhimurium (*S.* Typhimurium), so called because as well as causing a self-limiting gastroenteritis in humans resulting in fever and diarrhoea, it causes, as the name suggests Typhoid like symptoms in mice. *S.* Typhimurium SL1344 is used throughout this thesis and referred to as *Salmonella*, unless otherwise stated.

1.2 Sources of contamination

A natural *Salmonella* infection progresses post oral infection. The origin of infection can be from numerous sources. Approximately 95% of infections are associated with the eating of contaminated foodstuffs, this can include raw or undercooked meat either from an infected animal or one that has become contaminated post slaughter, eggs from infected chickens, and unpasteurised milk (Mead et al. 1999). There are also non-animal foods which can be sources of contamination, often due to the plants having been fertilized with contaminated water. Salad crops and other foods which are eaten raw are therefore common sources, this is due to the fact that they are poorly washed and not cooked before eating, so any bacteria which have adhered to the leaves will remain (L Plym and Wierup 2006, Lynch, Tauxe and Hedberg 2009, Islam et al. 2004, Henao-Herreño et al. 2016, Ibenyassine et al. 2007).

Salmonella is frequently found in environmental water samples, soil, and sediment. A review on water-borne *Salmonella* infections highlighted that the majority of papers looking at infections from this source were from Asia, and very few were from the other endemic regions of Africa and Central/Southern America (Threlfall 2002). These studies have highlighted the effect that lack of chlorination, equipment failures and back-siphonage in the water obtained from well-water, unboiled spring water and piped water of these areas have on infection numbers.

1.3 Route of infection

Once an individual has consumed the bacteria, since it has the ability to withstand high pH (Foster and Hall 1991, Garcia-del Portillo, Foster and Finlay 1993), it travels through the stomach and to the small intestine. Here it preferentially invades the epithelial cells of the small intestine (terminal ileum) or enters into the immune system by passing through M-cells which filter the gut lumen and transport foreign bodies to the Peyer's patches where they can be taken up by macrophages (Jones, Ghori and Falkow 1994, Galán and Curtiss 1989). Internalisation into macrophages occurs via the process of phagocytosis, dependent on the recognition of specific molecules by receptors on the surface of the macrophage. Inside the macrophage, *Salmonella* can replicate and in the case of typhodal strains disseminate and cause systemic infection

of other organs such as the spleen and liver (Alvarez-Ordóñez et al. 2011, Dougan et al. 2011, Mastroeni and Grant 2013, Carter and Collins 1974).

1.4 Disease Types

1.4.1 Gastroenteritis

The most common *Salmonella* infection in humans is Salmonellosis, which causes a gastrointestinal infection. A number of different *S. enterica* subspecies can lead to Salmonellosis in humans with around 20 serovars causing approximately 70% of cases, with the top three, *S. enterica* Typhimurium, Enteritidis and Newport causing 40% of infections. Symptoms of the infection usually occur 8-72 hours after ingestion of contaminated material and include inflammation of the intestine, abdominal cramping, vomiting and, diarrhea or constipation. In the large majority of cases the infection will be self-limiting and will be eradicated by the host immune system in 2-5 days with no requirement for treatment (Hohmann 2001). However, those under 5 or over 65 and immunocompromised patients may require treatment, which could either include hospitalisation in order to replace lost fluids or, antibiotics (Gordon 2008, Wen, Best and Nourse 2017, Musil et al. 2016). While Salmonellosis is a worldwide disease, the rates of infections vary between countries based on levels of contaminated food and water. A 2010 study estimates that there are 93.8 million cases of *Salmonella* induced gastroenteritis globally each year, with 155,000 (0.17%) of these resulting in death (Majowicz et al. 2010). While the cost to life of Salmonellosis is therefore quite low there is a large economic cost due to loss of productivity.

1.4.2 Enteric fever

In humans a systemic *Salmonella* infection is known as enteric fever and includes both typhoid fever and paratyphoid fever caused by *S.* Typhi and *S.* Paratyphi serovars. Symptoms of typhoid fever start similarly to Salmonellosis, with abdominal cramps, vomiting and diarrhea/constipation but combined with a high fever. Progression of the infection includes dissemination and subsequent inflammation of the liver and spleen, coupled with weight loss and severe confusion. If left untreated the infection can cause

either internal bleeding in the digestive tract or peroration of the digestive tract, this can result in sepsis and lead to multi organ failure which can be fatal.

Accurate data for the numbers of typhoid fever cases are unavailable, however estimates put the global total instances of typhoid between 11.9-20 million with about 1% of these, 129-200,000, resulting in death. In addition to this there are an estimated 5 million paratyphoid cases annually. (Crump, Luby and Mintz 2004, Crump 2014, Mogasale et al. 2014)

Typhoid fever must be treated with antibiotics in order to stop the infection spreading and becoming fatal. Therefore, in addition to the large number of deaths caused by the infection there is a large economic cost. Current vaccines and antibiotics and the issues faced in these areas are discussed in 1.5 Current Treatment.

1.4.3 Chronic carrier stage

Chronic carriers of typhoid fever are those who are infected with *S.* Typhi, appear to recover and are then symptomless and appear healthy. In these patients, the bacteria colonise the gall bladder, where they are protected from the host immune defense. From here the bacteria can be periodically released into the bile and excreted and hence a chronic carrier can go on to infect other patients without being aware they are doing so or that they are even infected. This is of particular concern in regions where there is poor sanitation and drinking water can become contaminated with human waste (Gonzalez-Escobedo, Marshall and Gunn 2011).

1.4.4 Invasive Non-typhoidal *Salmonella*

Invasive non-typhoidal *Salmonella* (iNTS) are non typhoidal strains, commonly the two serovars primarily responsible for gastroenteritis, *S.* Typhimurium or *S.* Enteritidis, which can cause a more typhoid-like infection. These strains are more resistant to the host-mediated killing by macrophages and hence are able to enter the bloodstream causing a systemic spread of the bacteria (Feasey et al. 2012). This disease has emerged in Africa, commonly developing into invasive diseases in individuals already suffering from malaria or HIV (Okoro et al. 2012). Between 22-25% of African patients succumb to a iNTS infection, much higher percentages than the global deaths from

typhoid fever, approximately 1% (Gordon et al. 2008). The link between HIV positive patients and those suffering from a iNTS disease is extremely high, with 95% of adults presenting with iNTS being HIV positive – this is not a link seen for Typhoid fever (Kankwatira, Mwafulirwa and Gordon 2004). The development of this disease in HIV positive individuals is due to abnormalities in the host not mutations of the bacteria. Three key defects have been described: a weakened gut mucosal layer which leads to increased dissemination from the gut; dysregulation of cytokines within immune cells; and impaired serum killing of the bacteria (Feasey, 2012).

1.5 Current Treatment

1.5.1 Antibiotic use and resistance

Typhoid fever is seen more in the developing world, partly due to poorer sanitation in these areas leading to acquisition of the infection through contaminated food or water, and partly due to the emergence of multi-drug resistant (MDR) strains in these areas.

To avoid fatality, patients with typhoid fever require treatment with antibiotics. The most common, 'first-line' antibiotics used to treat typhoid fever are chloramphenicol, amplicillin and trimethoprim; however, since 1989, strains of both *Salmonella* Typhi and Paratyphi are becoming increasingly resistant to these drugs, and these multi-drug-resistant (MDR) strains have been the cause of large outbreaks (Glynn et al. 1998, Rahman et al. 2014, Wong et al. 2015, Wirth 2015).

It is thought that in the Global North these MDR strains developed in the animal agriculture sector, where antibiotics are frequently and inappropriately used, before spreading to humans through contaminated foodstuffs (Marshall and Levy 2011, Holmberg et al. 1984, Helke et al. 2017).

The current antibiotic of choice is ciprofloxacin, however strains have been isolated which also show resistance to this drug (Threlfall 2002, Rowe, Ward and Threlfall 1997). This is becoming a rapidly increasing problem in the commonly endemic regions of the Global South but also due to individuals travelling more frequently to these regions, there are increasing reports of patients in the Global North with MDR *Salmonella* infections.

Along with methods aimed at reducing contamination and therefore cases of typhoid fever, new antibiotics are required to target the pressing issue of severe MDR *Salmonella.*

1.5.2 Vaccines

Due to the severity and increasingly hard to treat strains of *Salmonella* there is an increase in pressure on the availability of a long lasting vaccine.

A vaccine for typhoid fever was first developed in 1916 and relied on heat-killed *Salmonella* Typhi cells to produce a parenteral whole cell vaccine. This vaccine was reasonably effective, protecting 51 to 88% of those treated for up to 7 years. However, there were side effects that came with vaccination, including fever in up to 30% of cases, and headaches and severe localised pain in up to 35% (Guzman et al. 2006).

A modern vaccine was generated using purified, non-denatured, Vi capsular polysaccharide of *S.* Typhi. The Vi capsular antigen is encoded on the *Salmonella* pathogenicity island 7 (SPI-7), which is absent in the non-typhoid *Salmonella* typhimurium, and is known to be acutely important for virulence (Tran et al. 2010). The Vi vaccine is produced in multiple forms, Typhim Vi® (Sanofi Pasteur), Typherix® (GSK) and Typbar Vi® (Bharat Biotech) and has a reported efficiency of between 64 and 72% (Klugman et al. 1987, Acharya et al. 1987) with minimal side effects, the vaccine must be boosted every 2 years. In addition, there is a conjugated form of the vaccine which provides vaccination against tetanus in addition to typhoid, Typbar TCV®.

The other current vaccine is a live attenuated vaccine; this offers multiple benefits. It can be orally administered which both means no hazardous materials such as needles are produced and it can be easily administered not just by medical professionals. Importantly they can induce mucosal, cellular and humoral immune responses. Since live attenuated vaccines have the theoretical ability to revert to a fully virulent strain it is important that the attenuation is produced via multiple well defined mutations.

The Ty21a vaccine is an attenuated strain of *S.* Typhi Ty2 and contains a mutation in GalE and lacks the Vi antigen (Formal et al. 1981). The GalE mutation results in the strain being unable to produce the enzyme responsible for the reversible conversion of UDP-glucose to UDP-galactose, the accumulation of UDP-galactose in the

cytoplasm results in cell lysis (Germanier and Füer 1975). This strain also has numerous other mutations including in the gene encoding RpoS which is important for stress responses. This vaccine is currently in use as Vivotif® (PaxVax Corporation), which is administered as three or four capsule pills on alternate days, with protection being achieved 7 days after the final dose. High rates of efficiency and tolerability are seen with this vaccine, with up to 87% efficiency for up to 7 years and suggested re-immunisation after 5 years (Jin et al. 2017, Levine et al. 2007). Additionally, Ty21a has been shown to provide a level of protection against *S.* Paratyphoid B, which doesn't possess the Vi-antigen and therefore Vi polysaccharide based vaccines are ineffective, however use of both vaccines together has been heralded as a way to increase the immune response (Pakkanen et al. 2015).

One potential target for vaccine development is the recently discovered *Salmonella* Typhi toxin. This toxin is only found in *S.* Typhi and *S.* Paratyphi – two human host-restricted strains which cause enteric fever. Since the toxin is only present in these strains human studies will need to be carried out in order to know exactly what role the toxin plays in virulence and whether it can therefore be targeted as a preventative strategy; however the presence of the toxin has been linked to the progression of the disease and therefore it is expected that an antitoxin immune response should be able to be utlised to protect against Typhoid fever (Galán 2016).

There are many aspects to an ideal typhoid vaccine and each presents its own problems. Ideally a vaccine would protect against the range of *Salmonella* serovars which are responsible for invasive infections worldwide, these have been highlighted as *Salmonella* Typhi, *Salmonella* Paratyphi A, *Salmonella* Paratyphi B, *Salmonella* Typhimurium, *Salmonella* Enteritidis and *Salmonella* Choleraesuis (Tennant and Levine 2015). In addition, the current vaccines cannot be administered to the very young and this would be highly beneficial as the infection is more dangerous in these individuals (Crump and Mintz 2010). The live attenuated vaccines offer the best immune response but come with a risk of reversion to a virulent state, while this can be avoided with careful combinations of mutations, this requires extensive research (Spreng, Dietrich and Weidinger 2006).

1.6 Immune defenses and responses, and infection methods

Initial *Salmonella* infection results in the inflammation of local tissue via the presence of cell membrane components including lipopolysaccharide (LPS). This inflammation results in the recruitment of cytokines such as interferon-γ (IFN-γ) or tumor necrosis factor-α (TNF-α) which are required to activate macrophages (Arango Duque and Descoteaux 2014, Hu and Ivashkiv 2009, Duluc et al. 2009). As highlighted in the 1.3 Routes of infection section, it is important for the progression of *Salmonella* disease that the bacteria can survive within phagocytic cells including macrophages, as these phagocytes are central to the control of infection, being critical for survival in mice infected with *Salmonella*. Macrophages employ multiple antibacterial mechanisms in order to attempt to kill any bacteria that they have engulfs. This includes the release of reactive oxygen species (ROS) and the release of nitric oxide (NO) (Lowenstein et al. 1993). The inducible nitric oxide synthase, iNOS, is specific to macrophages and is responsible for their nitric oxide production (Aktan 2004).

1.6.1 *Salmonella* Pathogenicity Islands

The pathogenicity of *Salmonella* is largely down to regions of the genome referred to as *Salmonella* pathogenicity islands (Lostroh and Lee 2001). These islands contain the proteins required, and regulatory elements, for a specific virulence phenotype. These islands are highly conserved between the numerous *Salmonella* serovars and due to their lower GC content than the rest of the genome are thought to have been acquired via horizontal gene transfer (Li et al. 1995).

Salmonella pathogenicity islands 1 and 2 (SPI-1 and SPI-2) are found in all serovars in the *Salmonella enterica* species (Ochman and Groisman 1996). These SPIs encode type III secretion systems (TTSS), which allow for the transfer of bacterial proteins into the host cell. TTSSs are needle-like structures which span the bacterial envelope (Waterman and Holden 2003). The proteins injected by SPI-1 interfere with macrophage biochemistry and physiology and therefore vital processes, having effects including initiating the uptake of the bacteria. SPI-2 then promotes survival within the cell and causes apoptosis of macrophages.

1.6.1.1 *Salmonella* Pathogenicity Island 1

SPI-1 is 40kb in length, contains 29 genes, and is primarily known to be vital for the uptake by intestinal epithelial cells, which are not usually phagocytic (Zhou and Galán 2001). SPI-1 is important for the survival of all *Salmonella* within the intestine whether the particular serovar causes an enteric or a systemic infection (Lostroh and Lee 2001). Indeed while SPI-1 has been shown to be required for an orally-progressed infection it is non-essential for a systemic infection (Marcus et al. 2000, Jones et al. 1994). There are at least 13 proteins delivered by the SPI-1 machinery. Three important members of this group are SopB, SipA and SipC which are required for the rearrangement of actin in the host cell which results in the uptake of the bacteria (Raffatellu et al. 2005). The main regulatory trigger for SPI-1 is a change in pH that occur between the stomach and small intestine. HilA, HilC, HilD and InvF are the main regulators of SPI-1 (Eichelberg and Galán 1999). HilA is a member of the OmpR family of regulators and is known to be of particular importance for the regulation of SPI-1 (Bajaj, Hwang and Lee 1995).

1.6.1.2 *Salmonella* Pathogenicity Island 2

Once engulfed within a cell the large phagosome quickly reduces, within hours, to the *Salmonella* containing vacuole (SCV). SPI-2 behaves similarly to SPI-1 and inserts effector proteins across the SCV membrane into the cell cytosol via the translocon pore (Guignot and Tran Van Nhieu 2016). SPI-2 has four main functions, the acquisition of proteins from the host cell, the movement towards the nucleus and golgi apparatus, bacterial replication within the SCV and prevention of the binding of iNOS to the SCV reducing RNS exposure (Haraga, Ohlson and Miller 2008, Figueira and Holden 2012).

The positioning of the SCV near to the Golgi apparatus has been shown to be associated with an increase in bacterial growth. The effectors SseG, SseF and SifA are particularly important for this association of the SCV with the golgi (Ramsden et al. 2007). Myosin II, an actin-based motor, has also been shown to be important for this movement (Wasylnka et al. 2008, Steele-Mortimer 2008). A large number of effectors function to maintain the SCV, including SifA, PipB2, SseJ and SopD2, this

maintainance is vital for the ability of Salmonella to replicate within the host cell (Haraga et al. 2008, Waterman and Holden 2003, Cirillo et al. 1998).

Alteration of iNOS locality by the SCV is considered to be another SPI-2 dependent process, which in turn reduces the amount of RNS which target the bacteria; with peroxynitirite being excluded from the SCV via SPI-2 effectors. The significance of this role of SPI-2 has been demonstrated with the reduction in a virulence defect in $iNOS^{-/-}$ mice (Chakravortty, Hansen-Wester and Hensel 2002, Waterman and Holden 2003).

NRAMP1 is a macrophage transmembrane protein which is key to the killing of *Salmonella* by the macrophage and controls bacterial growth in the reticuloendothelial system. This has been well studied in the mouse model but a role for *NRAMPp1* in human infections has been harder to confirm (Dunstan et al. 2001, Mastroeni and Sheppard 2004).

1.7 Global microbial N-cycle

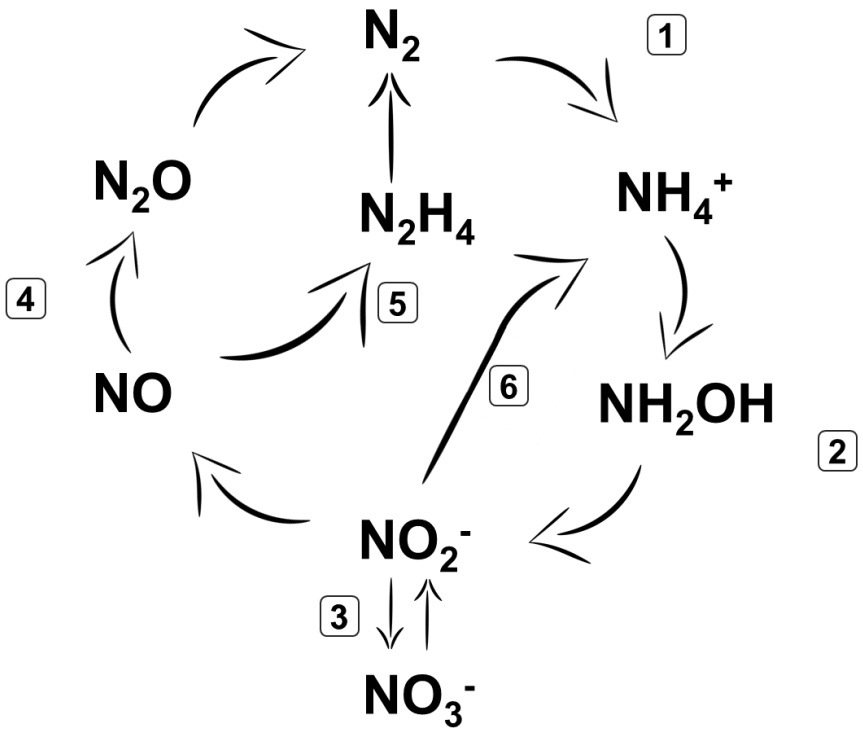


Figure 1 **The microbial Nitrogen Cycle.** Nitrogen Fixation (1), Aerobic Ammonia Oxidation/Nitrification (2), Nitrite Oxidation (3), Denitrification (4), Annamox (5), Dissimilatory Nitrate Reduction Pathway (6)

The nitrogen cycle is one of the key nutrient cycles in the environment; and microbes play a large role in this. All organisms rely on nitrogen for the synthesis of nucleic acids and proteins as well as many other essential compounds. The nitrogen cycle exists in 6 main parts, demonstrated in Figure 1.

In the atmosphere nitrogen exists as dinitrogen gas (N_2), an inert form of nitrogen. This therefore cannot be taken up by plants and so the nitrogen must be 'fixed' into ammonia (NH4+). This process is primarily undertaken by bacteria along with archaea. Nitrogen can also be fixed by lightning, but at an insignificant level (Ferguson and Libby 1971).

Nitrogen fixation is carried out by Diazotrophs, bacteria and archaea which are able to grow without the presence of fixed nitrogen. Some plants have symbiotic relationships with nitrogen fixing bacteria, commonly in the root nodules of legumes where Rhizobia are the most abundant. Some Cyanobacteria also form symbiotic relationships with plants such as ferns and fungi including lichens, these bacteria produce oxygen as a by-product of the process (Berman-Frank, Lundgren and Falkowski 2003). In traditional farming, fields would be cycled through rotations of food crops and legumes, often clover, these legumes then are left to rot on the fields and the captured nitrogen is released into the soil (Wang et al. 2014, Zhang et al. 2015). However, in modern farming practices this takes too long and is therefore not an efficient way to ensure the food crops are getting enough nitrogen and therefore nitrogen fertilisers are added. The Haber Bosch process requires very high pressures and temperatures to fix nitrogen from air mixed with natural gas (Sutton et al. 2011).

The next stage of the cycle is nitrification, where ammonium is oxidized via hydroxylamine, NH_2OH, to nitrite (NO_2^-), and subsequently to nitrate (NO_3^-). The first step is performed by ammonia-oxidising bacteria (AOB), primarily *Nitrosomonas* and *Nitrosococcus* and the ammonia-oxidising archaea (AOA), *Nitrosopumilus maritimus* and *Nitrososphaera viennensis* (Martens-Habbena et al. 2009). Nitrification can lead to issues with nitrogen run off and associated eutrophication, as nitrite is more soluble than ammonium (Conley et al. 2009).

Nitrite can then be returned to ammonia, via the dissimilatory nitrate reduction to ammonium (DNRA) pathway, to N_2 via N_2H_4 by the anammox pathway, or to N_2 via denitrification (Jetten 2008, Jetten et al. 2009). Traditionally little consideration has been given to the impact of the dissimilatory nitrate reduction to ammonium pathway to global nitrogen cycling; however, many soil bacteria have the ability to perform DNRA and is it thought to be the dominant pathway for nitrite cycling in some environments (Giblin et al, 2013).

Denitrification is the anaerobic reduction of nitrate (NO_3^-) via nitrite (NO_2^-) nitric oxide (NO) and nitrous oxide (N_2O) to dinitrogen (N_2). This process has been well studied in numerous bacteria, with the best understanding in the soil dwelling bacteria *Paracoccus denitrificans*. Research in this field has focused on the truncation of the pathway and subsequent release of the potent greenhouse gas N_2O into the atmosphere, and ways this could be reduced (Sullivan et al. 2013).

The nitrous oxide reductase, NosZ, is responsible for the conversion of nitrous oxide to dinitrogen gas and the completion of the nitrogen cycle. There are multiple factors known to affect the function of NosZ in agricultural soil dwelling bacteria, and hence the levels of N_2O which are successfully reduced. These include the amount of nitrogen fertilizer added to soils, water content, oxygen levels, nutrient availability, temperature and pH (Sahrawat, 1986(Sullivan et al. 2013).

1.8 Denitrification in *Salmonella*

Salmonella, along with other Enterobacteriaceae also undertake denitrification. However, unlike classical denitrifiers, the final step in the pathway, the reduction of N_2O to N_2 is missing, we therefore refer to denitrification in *Salmonella* as truncated denitrification (Rowley et al. 2012). This process is important for *Salmonella* in anaerobic or microoxic conditions because as facultative anaerobes they can use alternative electron acceptors, including nitrate, in order to respire.

The first step in this process, the reduction of NO_3 to NO_2 either uses the membrane-bound dissimilatory nitrate reducatase, Nar in the cytoplasm, or the periplasmic dissimilatory nitrate reductase, Nap. There are two membrane bound systems, that encoded by *narGHJI*, which functions anaerobically, and that by *narZYWV*, which is active aerobically, and a single periplasmic system, encoded by *napFDAGHBC*. (Rowley et al. 2012) NarG accounts for about 98% of nitrate reduction. In addition to nitrate reduction, NarG is able to catalyse the reduction of nitrite if no nitrate is present. The other enzyme responsible for this step is Nir, either NirK, the cytochrome cd1, cu-containing nitrite reductase (Gilberthorpe and Poole 2008) or a siroheme-containing nitrite reductase (NirB), *Salmonella* only possesses NirB.Ideally, to prevent NO accumulation, the NO-generating and NO-consuming processes should function in a concerted fashion to form N_2O, which until recently was considered to be less cytotoxic (discussed in 1.11 Nitrous Oxide).

The reduction of nitric oxide will be further discussed in 1.10 NO detoxification, and can take three forms, oxidation to nitrate, or reduction to ammonium or N_2O. There is no enzyme present in the entire Enterobacteriaceae family which reduces N_2O and hence in *Salmonella* denitrification is truncated at this stage.

Exposure to nitrate and nitrite in low oxygen conditions may occur outside the host but also in the mammalian intestine, as part of the host-generated inflammatory response (Lopez et al, 2012). Inflammation is generally thought to be a way that the host fights a pathogen, however this host generated nitrate has been shown to provide a growth advantage to members of the Enterobacteriaceae family including *E. coli* and *Salmonella.* The gut microbiota, which play many important roles including providing pathogen colonization resistance, is primarily made up of fermenting microbes which would be unable to use the nitrate as an electron source for respiration (Guarner and Malagelada 2003, Guarner 2006, Tremaroli and Bäckhed 2012). This phenomenon has been demonstrated multiple times, suggesting the ability of *Salmonella* to respire on nitrate and to trigger the host to produce this nitrate, is of key importance to its survival and virulence (Winter et al. 2013).

SopE, a type III effector protein, has been shown to increase the severity of inflammation in the intestine via Rho GTPase mediated caspase-1 activation and consequent release of the cytokine IL-1β (Müller et al. 2009). SipA is also delivered by a type III secretion system and plays a role in pathogen-mediated inflammation (Higashide et al. 2002, Stecher et al. 2007)

1.9 Toxicity of Nitric Oxide

Nitric oxide plays many roles in the human body including as a neuronal signaling molecule and as a regulator of vasodilation of blood vessels and hence blood pressure (Irshad and Chaudhuri 2002). Indeed, the latter of these functions has led to the increased use of nitric oxide as an athletic supplement, although studies on the benefits of these are inconclusive (Bescós et al. 2012). Conversely at the same time there is growing industry based around antioxidants designed to reduce RNS and ROS (Devasagayam et al. 2004).

As a free radical, nitric oxide (NO) has cytostatic and cytotoxic antibacterial activity in both aerobic and anaerobic environments, causing widespread damage to numerous cellular targets. Targets of this damage include DNA, proteins (both fully formed proteins and via amino acid targeting) and transcriptional regulators (Fang 2004). NO

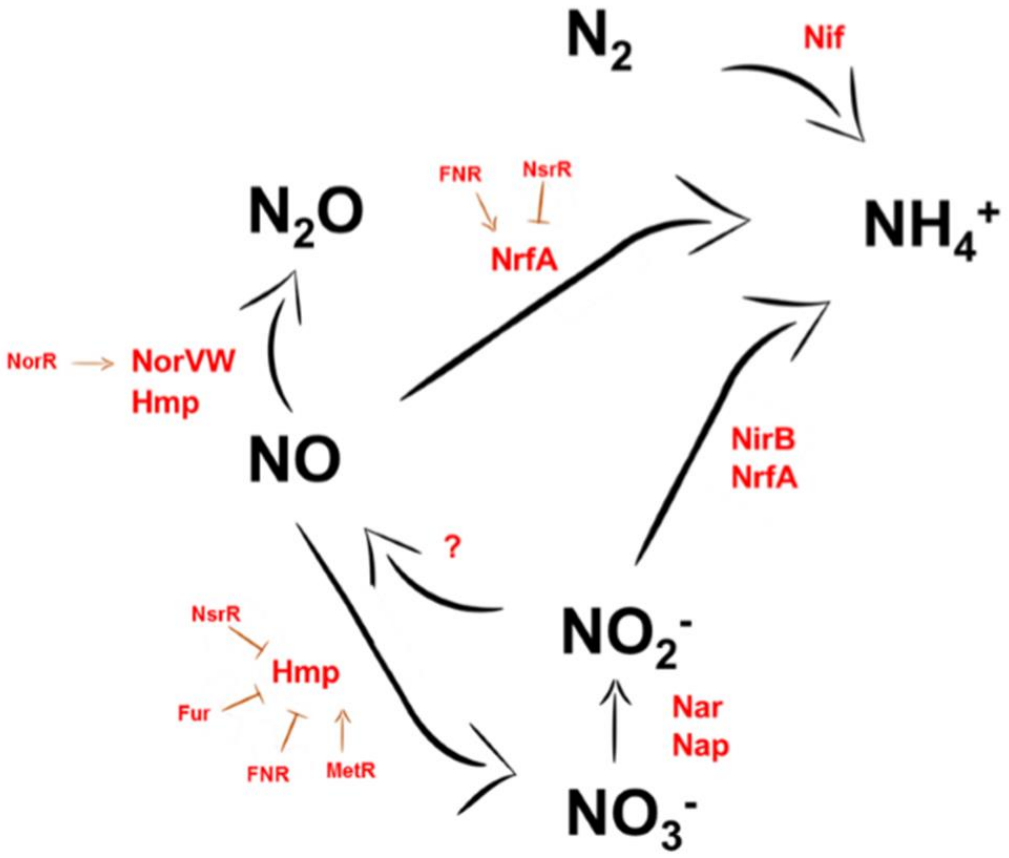
is highly reactive, and can form other toxic reactive nitrogen species (RNS), such as peroxynitrite (Brunelli, Crow and Beckman 1995).

Salmonella is exposed to RNS at different locations during its infectious cycle in vertebrate hosts (Prior et al. 2009). Nitrogen species are found distributed throughout the length of the gastrointestinal tract, representing a serious problem for enteric pathogens. The acidity of the stomach converts dietary and salivary nitrite to nitrous acid, and subsequently by disproportionation to other nitrogen species, including NO. The endogenous production of nitric oxide via the denitrification pathway has already been mentioned in 1.8 Denitrification in *Salmonella*.

Numerous cell types including leukocytes and hepatocytes can produce NO via the conversion of L-arginine to L-citrulline. Macrophages form a crucial part of the early response of the immune system to attack by pathogens, and production of NO is a vital part of the macrophage armory. Activated macrophages produce micromolar concentrations of NO through expression of inducible nitrous oxide synthase (iNOS), in response to inflammatory cytokines (IL-1, TNF-α, IFN-γ) and bacterial lipopolysaccharide (LPS) (De Groote and Fang 1995). The release of nitric oxide by macrophages has been seen to occur within two hours of phagocytosis and been shown to clear 99% of *S.* Typhimurium within six hours (Vazquez-Torres et al. 2000).

The iNOS mediated production of NO is an important defense mechanism against *S*. Typhimurium infection, with mice lacking functional alleles of *iNOS* significantly more susceptible to *Salmonella* infection (Alam et al. 2002, Mastroeni et al. 2000). Paradoxically, host derived NO and the ensuing pro-inflammatory cascade during enteritis has also been shown to be of benefit to *Salmonella*, in outcompeting the commensal flora of the intestine (Stecher et al. 2007).

1.10 NO detoxification



Enzymes	Regulators
Hmp flavohaemoglobin	Fur ferric uptake regulator
Nar membrane-bound dissimilatory nitrate reductase	FNR fumarate and nitrate reductase regulator
Nap periplasmic dissimilatory nitrate reductase	MetR methionine repressor
NirB siroheme containing nitrite reductase	NsrR nitric oxide sensitive repressor
NrfA cytochrome c nitrite reductase	NorR nitric oxide reductase regulator
Nif nitrogenase	
NorVW anaerobic nitric oxide reductase flavorubredoxin	

Figure 2 **Nitrogen cycle in *Salmonella*.** Enzymes and regulators involved are shown in red, positive regulators are indicated by arrows and repressors by perpendicular lines. Adapted from (Arkenberg et al. 2011)

Two key enzymes in detoxification of NO are flavohemoglobin (HmpA) and flavorubredoxin (NorVW) (Mills et al, 2008), which convert nitric oxide to nitrous oxide under anoxic conditions. This represents the conversion of a potent cytotoxin to a potent greenhouse gas. In order to better understand the underlying metabolic driving force for intracellular nitric oxide production under nitrate-sufficient / glycerol limited and nitrate-limited / glycerol sufficient conditions in continuous cultures of *Salmonella* Typhimurium have been studied (Rowley et al 2013) and transcription of genes of the nitric oxide responsive NsrR regulon were used as a qualitative reporter of intracellular NO generation. Since nitric oxide is detoxified by conversion to nitrous oxide in the cytoplasm, direct measurement of nitric oxide released by bacteria will grossly underestimate the actual level produced intracellularly by nitrate metabolism. *S.* Typhimurium cannot reduce nitrous oxide and so nitrous oxide production has been measured as a more quantitative measure of the fraction of nitrate metabolized that forms nitric oxide. Under nitrate-sufficient / nitrite-sufficient / glycerol-limited conditions this can be a remarkable ~15% and 100% ~of the total nitrate and nitrite consumed in the bioreactor, respectively. This decreases to <0.1% under nitrate-limited / nitrite-limited / glycerol-sufficient conditions.

1.10.1 Mechanisms for NO detoxification

As a critical component of intracellular survival much more focus has been given to the detoxification of exogenous, rather than endogenous NO, with several systems implicated in the enzymatic detoxification of NO. Well-characterized enzymes with the ability to metabolize NO include the soluble flavohaemoglobin HmpA, the di-iron centred flavorubredoxin NorV with its NADH- dependant oxidoreductase NorW (NorVW) and the cytochrome c nitrite reductase NrfA. NorVW and NrfA are only active under anaerobic or micro-oxic conditions. NorVW reduces NO to N_2O, whereas NrfA uses either NO or nitrite to form ammonia. Flavorubredoxin (NorV) is an oxygen-sensitive nitric oxide reductase which reduces NO to N_2O (Hutchings, Mandhana and Spiro 2002). Neither, NorV or NrfA are required for *Salmonella* survival in mice (Bang *et al.*, 2006).

HmpA has only a minor role in NO detoxification under anoxic conditions, where it converts NO to N_2O, but is the crucial enzyme when oxygen is present, reducing NO

to NO_3^-. Expression of *hmpA* in *Salmonella* has been shown to be induced by NO, and repressed by intracellular iron, an important mechanism by which detoxification of NO is accomplished without causing oxidative stress (Bang et al. 2006). Such NO detoxification is vital for survival in the presence of both oxidative and nitrosative stresses. *hmpA* is highly induced in *S.* Typhimurium inside macrophages (Eriksson et al. 2003), suggesting that HmpA is involved in the bacterial defence against the nitrosative burst. However, this is not the case in HeLa epithelial cells where *Salmonella* is not exposed to either oxidative or nitrosative stress (Hautefort et al. 2007). *hmpA* mutants are moderately attenuated in a C3H/HeN mouse virulence model (*ity^R)*, but not in C57/BL6 mice (*ity^S)* (Bang et al. 2006). C57/BL6 mice succumb to *S.* Typhimurium infection before they produce a nitrosative burst. A key regulator of HmpA expression is the **n**itric oxide-**s**ensitive **r**epressor NsrR. NsrR belongs to the Rrf2 family of transcriptional repressors and senses NO specifically by a [2Fe-2S] cluster. This assumption results from great similarities between NsrR and other [2Fe-2S] cluster containing members of the Rrf2 family like IscR or RirA (Schwartz *et al.,* 2001*)*. The presence of the [Fe-S] clusters makes the protein structure and binding prone to damage by NO so that genes repressed by NsrR, are de-repressed after exposure to NO. The NsrR regulon of *Salmonella* consists of at least *hmpA*, *ytfE*, *ygbA*, *hcp-hcr*, *yeaR-yoaG*, *nrfA*, *tehB* and STM1808 (Karlinsey et al. 2012). *ytfE* (*nipC*), is highly induced in *E. coli* under conditions of nitrosative stress, contains a di-iron centre of the histidine-carboxylate family and is involved in the repair of damaged [Fe-S] clusters. Mutation of *ytfE* in *E. coli* results in a strain that grows poorly under anaerobic respiratory conditions and that has an increased sensitivity to iron starvation (Justino et al., 2006). In *S*. Typhimurium, *yftE* expression is induced by nitrite and during RAW264.7 macrophage infection, although the *ytfE* mutant constructed did not display a growth defect under these and other inducing conditions. Most surprisingly given the NO sensitivity, a *ytfE* mutant has a lower LD_{50} post low dose oral infection of C57BL/6J mice than the isogenic parent. STM1808 is annotated on the *S*. Typhimurium genome as a putative cytoplasmic protein, but the conserved domain database identifies a DUF1971 superfamily domain within the protein sequence, possibly involved in tellurite resistance. During a bioinformatic screen for NsrR binding sites, an NsrR promoter box (ggtgtatattaaatacatc) was first predicted upstream of STM1808 (Rhodionov et al 2005). TehB and YeaR are other putative tellurite resistance proteins identified as NsrR regulated in *E. coli*, indicating a role for tellurite resistance proteins,

either directly or indirectly, in NO detoxification. In *S. Typhimurium* STM1808 was the most up-regulated gene during RNS stress at pH4.4. More recently deletion mutants of NsrR regulated genes were exposed to the NO-releasing compound Spermidine-NONOate in LB cultures in the presence of oxygen (Karlinsey et al 2012). Confirmation of *hmpA* sensitivity was demonstrated along with impaired growth of Δ*STM1808*. No sensitivity of other NsrR regulated genes was observed. In M9 minimal medium the sensitivity of strains increases. A contribution of HmpA, Hcp, YgbA and STM1808 is proposed to help with the aerobic resistance of *S*. Typhimurium against nitrosative stress (Karlinsey et al. 2012).

1.11 Nitrous Oxide

The end protect of *Salmonella's* truncated denitrification is nitrous oxide (N_2O). N_2O is most commonly thought of a potent greenhouse gas, with atmospheric concentrations rising and a radiation potential over 300 fold higher than carbon dioxide and a high stability meaning it can last for 120 years (Ravishankara, Daniel and Portmann 2009, Canfield, Glazer and Falkowski 2010). A large amount, over 60%, of the nitrous oxide released into the atmosphere comes from agricultural soils where nitrogen fertilisers are added to increase crop yield but simultaneously triggers denitrification in microbes, some of which under certain conditions do not undergo complete denitrification and release nitrous oxide.

There is therefore a focus on the part microbes have to play in the production of N_2O and potential mitigation strategies which could be employed to halt the increase of the gas in our atmosphere (Richardson et al. 2009). In some bactria, nitrous oxide reductase (NosZ) is responsible for the conversion of N_2O to inert N_2 gas and therefore the correct functioning of this enzyme is vital to the reduction of this gas in the atmosphere. One study unveiled the importance of extracellular copper concentration for the expression of *nosZ* as well as being important as a cofactor (Sullivan et al. 2013). A link was also made between N_2O release and vitamin B_{12}-dependent pathways, showing a cytotoxic effect of N_2O in the important denitrifier *Paracoccus denitrificans*, this will be further expanded upon in this thesis and discussed in 1.13.2 Methionine synthases and nitrous oxide.

A combination of studies by (Stremińska et al. 2012, Rowley et al. 2012) and Runkel et al (personal communication) has identified that nitrous oxide production by *Salmonella* Typhimurium cultured in nitrate sufficient/glycerol limited chemostats is an order of magnitude higher than that produced by related non-pathogenic *E. coli* strains (Figure 3). *E*. coli possesses the same denitrification mechanisms as *Salmonella*, or more importantly, lacks the same gene, nitrous oxide reductase and therefore similarly has a net release of nitrous oxide.

Based on these studies, in a recent review on the biological role of nitrite (Maia and Moura 2014) the authors queried why *Salmonella* produce a large level of both NO and N_2O. The question was raised that given the cytotoxic effects of NO why is it the case that is it being produced – is it to be reduced in order to produce N_2O and if so for what reason? This is discussed further in 1.13.2 Methionine synthases and nitrous oxide.

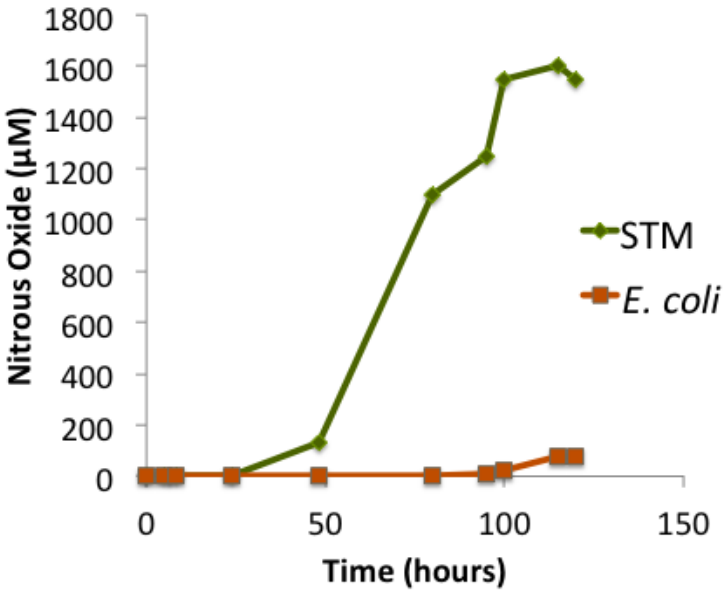


Figure 3 **Levels of N_2O produced by *S.* Typhimurium SL1344 and *E. coli* MG1655.** Strains grown in nitrate sufficient, glycerol limited minimal media in chemostats. (Runkel, personal communication).

1.12 Vitamin B_{12} and *Salmonella*

1.12.1 Vitamin B_{12} synthesis

Vitamin B_{12}, or cobalamin, is one of the most complex molecules in the natural world, it consists of a central corrin ring containing a cobalt ion. The synthesis of this vitamin is limited to bacteria and archaea; even though it is also required by animals. Due to the molecules complexity, over 30 genes are required for its complete synthesis, in *Salmonella* these genes are clustered together in the *cob* operon, which accounts for 1% of the genome (Martens et al. 2002, Jeter and Roth 1987). It is important to note that it has been reported that the *cob* operon is only activated under anaerobic conditions, and therefore the synthesis of vitamin B_{12} by *Salmonella* only occurs anaerobically (Jeter, 1984).

The synthesis of vitamin B_{12} is split into three parts. Part I is the conversion of uroporphyrinogen III (Uro III) to cobinamide. This involves the methylation of the porphyrin ring, amidation of carboxyl groups, removal of ring carbon, insertion and reduction of the cobalt and addition of the edenosyll moiety and aminopropanol side chain. Part II produces the lower axial ligand of the cobalt atom, by synthesizing dimethylbenzimisdazole (DMB). Part III covalently links the products of parts I and II, the combinamide and DMB with a phosphoribosyl moiety to complete the production of cobalamin (Roth et al. 1993).

Vitamin $B_{12,}$ produced by bacteria, including *Lactobacillus reuteri* is present in the human gut, but bacterial uptake is often limited due to the presence of an efficient host uptake system (LeBlanc et al. 2013). The absorption of Vitamin B_{12} in the human intestine has been studied since the 1950's (Booth and Mollin 1959, Cooper and Castle 1960). Absorption of B_{12} is reliant on a glycoprotein called intrinsic factor which is produced by the parietal cells of the stomach (Fedosov 2012, Fyfe et al. 2004, Moestrup 2006).

1.12.2 Vitamin B_{12} requirements

Vitamin B_{12} is required for a wide range of processes in both bacteria and animals. One common process is in the production of acetyl-CoA, an important molecule in the Krebs cycle; another is in ribonucleotide reduction which is important for DNA synthesis in bacteria; most enteric bacteria also rely on vitamin B_{12} in order to ferment 1,2-propandiol, ethanolamine and glycerol, which is important in anaerobic environments (Martens et al. 2002).

In humans there are two processes which rely on vitamin B_{12}, the folate dependent methylation of homocysteine to methionine and the production of succinyl-coenzyme A from methylmalonyl-coenzyme A (Fenech 2001). B_{12} deficiency in humans leads to an increase in homocysteine levels which can have serious implications in the risk of stroke and heart disease (Martens et al. 2002).

In *Salmonella* there are four vitamin B_{12} dependent enzymes: vitamin B_{12}-dependent methionine synthase, MetH; ethanolamine ammonia lyase, which degrades ethanolamine (Roof and Roth 1989); propanediol dehydratase which allows *Salmonella* to use propanediol as a carbon source; and queuosine synthase, which produces queuosine, a nucleoside found in four tRNAs which is not essential for growth under laboratory conditions (Frey et al. 1988).

Until 2001 (Price-Carter et al. 2001) it was thought that these processes were all non-essential, that is, that the deletion of vitamin B_{12} synthesis from *Salmonella* had no detrimental effect on *Salmonella* under any conditions tested. This therefore resulted in a B_{12} paradox – why would *Salmonella* have retained this large and energetically expensive operon if there is no physiological requirement for the end product? This paradox has been researched in recent years and some insights have been reported.

Tetrathionate is an alternative electron acceptor which is produced in the inflamed intestine via the reaction between reactive oxygen species and luminal sulphur compounds. *Salmonella* has the ability to respire on tetrathionate, an ability that enables it to outcompete the host microbiota (Winter et al. 2010). Tetrathionate allows *Salmonella* to grow on both ethanolamine and propanediol, both of which are vitamin B_{12} dependent processes (Price-Carter et al. 2001, Thiennimitr et al. 2011). Of note, Price Carter *et al* state that the utilisation of ethanolamine in order for *Salmonella* to

grow using tetrathionate are the only conditions they know of where *Salmonella* requires vitamin B_{12}.

1.13 Methionine synthesis

Methionine is an essential amino acid that can be produced by *Salmonella* via two enzymes, a vitamin B_{12} dependent (MetH) or vitamin B_{12} independent (MetE) methionine synthase. This is the only process in *Salmonella* that has both a vitamin B_{12} dependent and independent system. Both enzymes catalyse the transfer of a methyl group from methyltetrahydrofolate to homocysteine which leads to the production of H_4Folate and methionine (Banerjee and Matthews 1990, Drennan, Matthews and Ludwig 1994, Kräutler 2005). MetH uses methylcobalamin as the intermediate by which this methyl transfer occurs, whilst MetE requires folate (Schulte, Stauffer and Stauffer 1984). It has been reported that the vitamin B_{12} dependent enzyme, MetH, is 60 times more active than the alternative vitamin B_{12} independent enzyme, MetE (Smith and Neidhardt 1983). MetE is divergently transcribed to the MetR transitional regulator, whilst MetH is located in a completely different genomic loci (Figure 4).

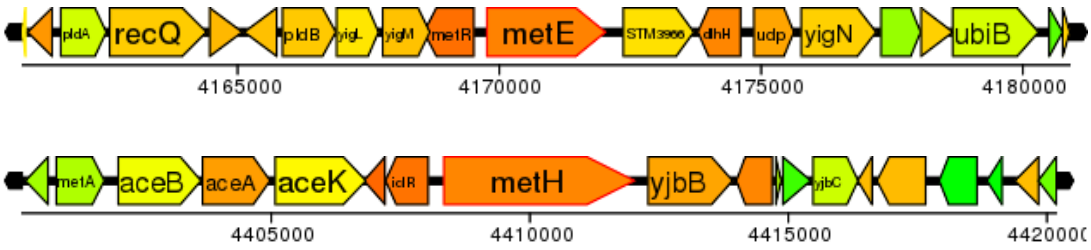


Figure 4 **Genomic location of *metH and metE, metR.*** Numbers indicate nucleotide position on the S. Typhimurium LT2 genome. Constructed in colibase.

It should therefore be the case that since *Salmonella* can only produce vitamin B_{12} under anaerobic conditions that *metE* mutants are methionine auxotrophs in the presence of oxygen and in the absence of exogenous vitamin B_{12} (Escalante-Semerena, Suh and Roth 1990). Jeter et al., showed that while *metE* mutants cannot

grow aerobically they can grow anaerobically even without exogenous vitamin B_{12} or methionine, due to their ability to synthesis the required vitamin B_{12} (Jeter, Olivera and Roth 1984). A double *metE cob* mutant, which relies on the vitamin B_{12} dependent MetH to synthesis methionine, and is lacking the proteins required to produce vitamin B_{12}, cannot grow aerobically or anaerobically.

In a study by Smith and Neidhardt in 1983 which looked at proteins in *E. coli* that are induced by oxygen, *metE* was identified as oxygen inducible and also stated that *metH* is inhibited by the presence of oxygen. This confirms the findings by Jeter et al., that a *metE* mutant cannot grow aerobically, since the remaining methionine synthase, *metH,* would be repressed (Jeter et al. 1984). Expression data produced by (Kröger et al. 2012), supports this report, with the caveat that this is nutrient rich media, which contains methionine.

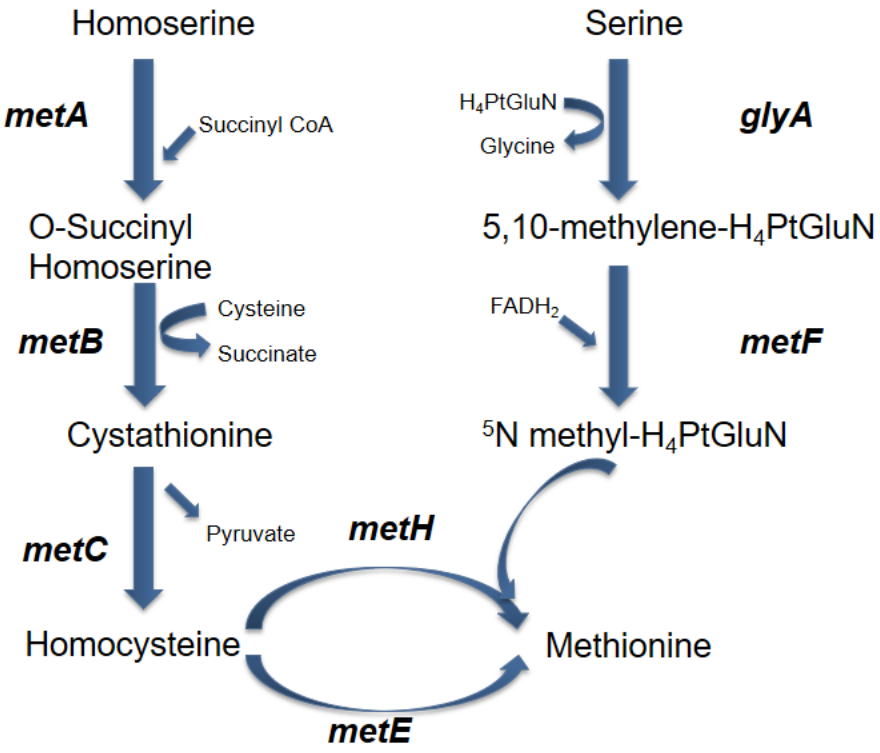


Figure 5 **Methionine synthesis in *Salmonella*.** The final stage converts homocysteine to methionine and can either be vitamin B_{12} dependent (*metH)* or independent (*metE*).

1.13.1 Regulation of methionine synthesis

Both *metE* and *metH* are regulated by MetR (Urbanowski et al. 1987, Urbanowski and Stauffer 1989). MetR belongs to the LysR family of helix-turn-helix transcriptional regulators, and binds as a dimer to a dyad sequence. Homocysteine, an intermediate in the biosynthesis of methionine, binds to MetR and enhances the activity of some MetR-activated promoters (*metE* and *glyA* (a serine hydroxymethyltransferase). The exception is *metH*, which is activated by MetR in the absence of homocysteine. There is some inconsistency in the literature as to how well MetR induces expression of *metH,* however in *E. coli*, *(Cai et al. 1989)* showed that MetR stimulated *metE* expression ~16-fold and *metH* expression ~8-fold. MetR also acts as a negative regulator of its own expression.

Expression of *metE,* as well as the other methionine biosynthetic genes, with the exception of *metH*, are repressed by the addition of high levels of methionine to the growth medium. This repression is mediated by a repressor protein, MetJ, and a co-repressor, S-adenosylmethionine (SAM or AdoMet). Vitamin B_{12} can repress the synthesis of MetE, mediated by the MetH holoenzyme, which contains a cobamide prosthetic group.

There have been reported differences between the regulation of methionine biosynthesis in *Salmonella* and *E. coli*. For example there is no MetJ binding site upstream of *metH* in *Salmonella*, and we should consider this when relying on the *E. coli* literature (Cai et al. 1989).

1.13.2 Methionine synthases and nitrous oxide

It was first reported in 1968 that nitrous oxide (N_2O) can bind to, and inactivate vitamin B_{12}. Since then this has been confirmed many times over due to the use of N_2O as an anaesthetic (Nunn 1987, Sanders, Weimann and Maze 2008, LASSEN et al. 1954). Vitamin B_{12} deficiency (hyperhomocysteinemia) is most commonly seen due to a lack of the vitamin in the diet (Stabler and Allen 2004). However, there are also many reports of the deficiency due to prolonged exposure to nitrous oxide, for instance in anesthesiologists or those that recreationally abuse the gas (Chiang et al. 2013).

In mice, rats and in humans (where there is only a MetH type vitamin B_{12} dependent methionine synthase) it has been shown that methionine levels drop after exposure to N_2O (Nunn 1987). This is due to N_2O binding to and depleting the vitamin B_{12} pool and thus MetH cannot function (Deacon et al. 1980).

In a similar scenario to that reported in mammalian cells, when nitrous oxide is present in high levels in the bacteria *Paracoccus denitrificans*, the production of methionine by MetH is inhibited due its requirement for vitamin B_{12}. However, due to the presence of a secondary methionine synthase, which does not require vitamin B_{12}, production of methionine can continue. It has been shown that under these conditions *metE* is upregulated in order to compensate for the functional loss of *metH* (Sullivan et al. 2013). It has also been previously shown in *Paracoccus denitrificans* that in conditions when N_2O accumulate (a *nosZ* mutant), a *metE* deletion strain (where MetH is the only enzyme available to produce methionine) has hampered growth.

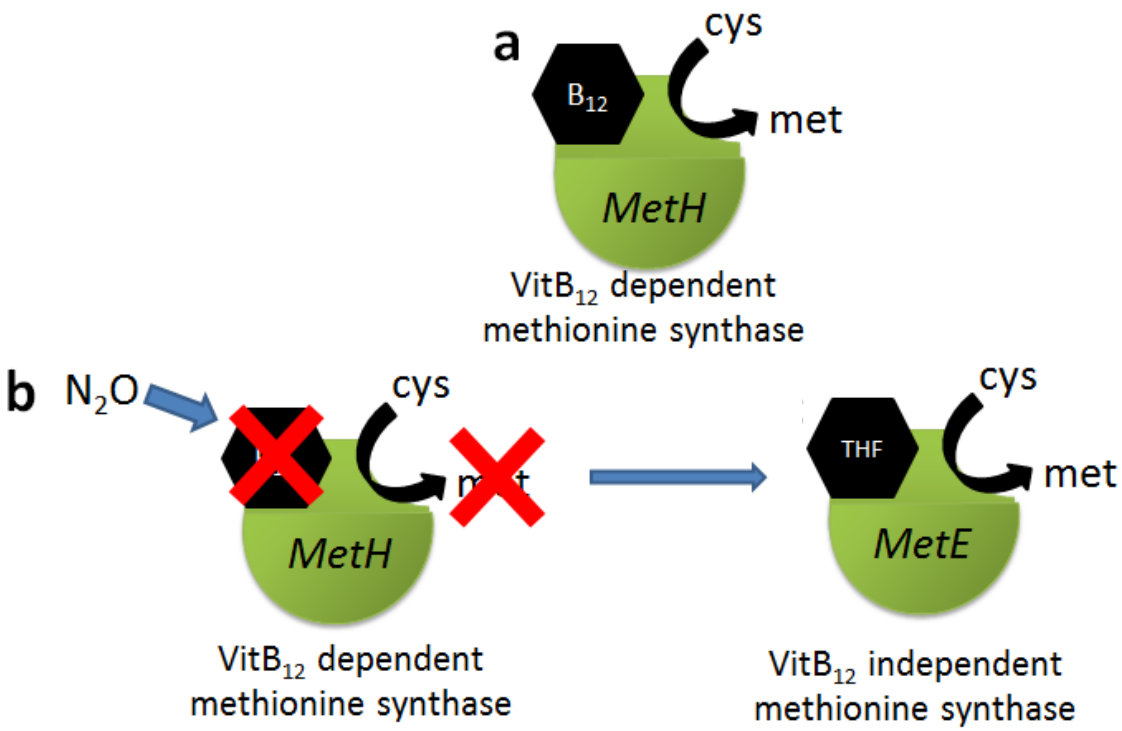


Figure 6 **Outline of the inhibition of N_2O on the production of methionine by MetH in *Paracoccus denitrificans*** A) In the absence of N_2O methionine is produced by MetH. B) In the presence of N_2O, vitamin B_{12} is targeted by N_2O and so methionine is produced by MetE not MetH.

There has been controversy in the past regarding whether or not nitric oxide as well as nitrous oxide can react with vitamin B_{12} but there are some studies, which demonstrate that nitric oxide has a similar inhibitory effect. It has been shown that NO can inhibit the synthesis of methionine by MetH, in rat hepatocytes (Nicolaou et al. 1996) and demonstrated spectroscopically that NO is able to interact directly with vitamin B_{12} and stop the methionine synthase activity in cultured rat muscle cells (Danishpajooh et al. 2001). Biochemical analysis has revealed that nitric oxide has a high affinity for cobalamin II, and interacts with cobalamin III at low pHs (Sharma et al. 2003).

1.4 Research Gap:

Salmonella is a pathogen which causes disease worldwide, with both a huge economical burden and cost to human life. The ever growing concerns of MDR strains are putting pressure on the discovery of new antibiotics to treat those infected and new vaccines to prevent disease. A new potential vaccine strain has many desired characteristics but to start with must have a range of mutations which allow it to survive within the host without being virulent. It is therefore required to build on the fundamental knowledge of processes *Salmonella* employs to contribute to its pathogenesis. One area where there are gaps in the research is in the nitrogen cycle. Nitric oxide is a highly toxic compound produced both by macrophages as part of the host response and by *Salmonella*'s own metabolism when growing on nitrate. Since *Salmonella* must be able to survive within macrophages in order for disease to progress effective detoxification of nitric oxide is therefore vital. While this topic is fairly well understood, particularly aerobically, there are potentially as yet undiscovered proteins which are involved in the anaerobic detoxification of nitric oxide.

One route for detoxification of nitric oxide is to nitrous oxide. It has been observed that *Salmonella* produces high levels of this gas, much higher levels than its close relative *E.* coli; it is however not clear why this is the case. Understanding why *Salmonella* releases this toxic gas in such large quantities is important to the overall understanding of the nitrogen cycle in *Salmonella*.

2 Materials and Methods

2.1 Strains & plasmids

The isogenic parent strain from which all other mutants used in this study are derived is *Salmonella enterica* serovar Typhimurium SL1344 (Hoiseth & Stocker, 1981). A list of all strains and plasmids used in this study can be found in Table 1 and 2 respectively.

Table 1 **A list of all strains used in this study, their genotypes and origins.**

Strain	**Genotype**	**Source**
SL1344	*S. enterica* serovar Typhimurium 4/74 hisG rpsL	(Hoiseth and Stocker 1981)
S. Cholerasuis	*Salmonella enterica* serovar Choleraesuis	Mark Roberts, personal communication
S. Dublin	*Salmonella enterica* serovar Dublin	Mark Roberts, personal communication
S. Gallinarum	*Salmonella enterica* serovar Gallinarum	Mark Roberts, personal communication
S. Pullorum	*Salmonella enterica* serovar Pullorum	Mark Roberts, personal communication
S. Enteritidis	*Salmonella enterica* serovar Enteritidis	Mark Roberts, personal communication
C. rodentium	*Citrobacter* rodentium	Gad Frankel, personal communication
GR0301	SL1344 *ΔcstA*::cmR	This Study
GR0303	SL1344 ΔytfE::kanR	This Study
	SL1344 ΔnorV::kanR	Mills et al, 2008

	SL1344 ΔnrfA::kanR	Mills et al, 2008
GR0306	SL1344 ΔnsrR	This Study
GR0308	SL1344 *ΔtehB*	This Study
GR0309	SL1344 ΔhmpA::cmR	This Study
GR0310	SL1344 ΔSTM1808	This Study
GR0311	SL1344 *ΔyeaR*	This Study
GR0312	SL1344 ΔSTM1808 *ΔtehB*::cmR *ΔyeaR*::kanR	This Study
GR0320	SL1344 *ΔmetE*::cmR	This Study
GR0321	SL1344 *ΔmetH*::kanR	This Study
GR0322	SL1344 *ΔmetR*::kanR	This Study
GR0323	SL1344 *ΔmetE*::cmR *ΔmetH*::kanR	This Study
GR0324	SL1344 *ΔpocR*::kanR	This Study
GR0325	SL1344 *ΔmetE*::cmR *ΔpocR*	This Study
GR0326	SL1344 *ΔmetE*::cmR ΔcobS	This Study

Table 2 **A list of all plasmids used in this study, their genotypes and origins.**

Plasmid	Genotype	Source
pCP20	Temperature sensitive replication and thermal induction of FLP synthesis, Amp^R, Cm^R	(Cherepanov and Wackernagel 1995)
pkD3	Amp^R, pANT-Sγ derivative containing a FRT-flanked Cm^R cassette	(Datsenko and Wanner 2000)
pkD4	Amp^R, pANT-Sγ derivative containing a FRT-flanked Kan^R cassette	(Datsenko and Wanner 2000)
pkD46	Amp^R, pINT-ts derivative containing a araC-P_{araB} and γ, β, exo genes	(Datsenko and Wanner 2000)

2.2 Media

2.2.1 Rich media

The following rich media are used in this study:

• Luria-Bertani (LB) broth:

10 g Tryptone, 5 g Yeast Extract, 10 g NaCl per 1 L distilled H_2O (dH_2O)

• Green plates for selection of non-lysogenic P22 transductants:

8 g Tryptone, 1g Yeast Extract, 5 g NaCl, 1.5% (w/v) agar per 1 L dH2O; addition of 21 mL of 40% (w/v) glucose (F/S), 25 mL of 2.5% (w/v) alizarin yellow G and 3.3 mL of 2% (w/v) aniline blue (F/S) after autoclaving.

2.2.2 Minimal media

M9 minimal media was used for all minimal media experiments composing of Na2HPO4 6 g/l, KH_2PO_4 3 g/l, NaCl 0.5 g/l, NH_4Cl 0.1 g/l to be autoclaved, and then additionally $MgSO_4$ 1mM, $CaCl_2$ 0.1mM. Either glucose, 55mM, or $NaNO_3$ 20mM and glycerol 5mM were added as indicated. When indicated methionine (30 µg/ml) or vitamin B_{12} (concentrations as indicated) were added.

Sterilization of media is achieved by autoclaving at 121°C for 15 min. Media was supplemented with Kanamycin (75 µg/mL), Chloramphenicol (10 µg/mL), Ampicillin (100 µg/mL), when appropriate.

2.2.3 Overnight cultures

Aerobic overnight cultures are prepared using 10 ml of the same media as to be used in the main experiment in a 25 ml universal, supplemented with appropriate antibiotics. The bacterial cultures are incubated overnight at 37°C with shaking at 200 rpm.

Anaerobic overnight cultures are prepared using the same media as to be used in main experiment, supplemented with appropriate antibiotics. A 50 ml falcon was filled to the top with media and sealed with parafilm. The bacterial cultures are incubated overnight at 37°C statically. When strains were grown in M9 minimal media, methionine (30 mg/ml) was added to ensure equal growth of all strains (including methionine auxotrophs); cultures were then washed twice with PBS prior to use.

2.4 Aerobic growth

Aerobic growth was conducted in 50 ml media in 250 ml conical flasks at 37°C with agitation or in 24 well plates in 1 ml media at 37°C with 10 seconds agitation every 15 minutes as indicated.

2.5 Anaerobic growth

Anaerobic growth was primarily conducted in 200 ml media in 250 ml Durans. After inoculation media was sparged with N_2 gas for 10 minutes to create anaerobic conditions. Cultures were incubated statically at 37°C. When the cultures were saturated with N_2O, first they underwent N_2 saturation and then 5 minutes of sparging with 99% N_2O.

Where indicated growth was conducted in 1 ml media in 24 well plates, 400 µl of mineral oil was gently pipetted on top of the media and the plates were sealed with a silicon sealant and parafilm. Cultures were incubated statically at 37°C in a SpectraMax Plus 384 Microplate Reader.

2.6 Mutant Construction

Deletion of genes was carried out using lambda red recombination as previously described (Datsenko and Wanner 2000), briefly this relies on amplifying an antibiotic gene cassette, either from plasmid pKD3 (Cm^R) or pKD4 (Kan^R), with homologous flanking regions to the gene of interest and transforming this into *S.* Typhimurium containing pKD46. This plasmid encodes the lambda red recombinase system which recognises the homologous region and exchanges the gene of interest with the antibiotic resistance cassette. Mutations were then moved to a clean SL1344 background via P22 bacteriophage transductions, and Green plates used to select for non-lysogenic colonies.

Primers used for construction and verification of mutants are listed in Table 3 and 4, PCR components and protocols are listed in Table 5 and 6. The four reactions carried out to confirm correct mutant construction were, forward and reverse externals for the gene of interest both on the 'mutant' and the WT, and forward external for the gene of interest and reverse internal for the relevant cassette, both on the 'mutant' and the WT. The former creates different size bands based on the difference between the length of the gene and the size of the cassette, and the later cannot form a product for the WT and confirms the cassette is inserted in the correct location for the mutant. Expected sizes for each reaction are given throughout this thesis for each gel image.

DNA for PCR was either obtained via QIAprep Spin Miniprep Kit (QIAGEN) according to manufacturer's instructions and eluted in 50 µl dH_2O, or alternatively via a colony boilate whereby a colony was suspended in 100 µl dH_2O, placed at 100 °C for 10 minutes, centrifuged at 13,000 rpm for 1 min and the supernatant used.

Table 3 **Primers used for mutant construction**

	Forward Primer	Reverse Primer
cobS	ATGAGTAAGCTGTTTTGGGCCATG CTCGCTTTTATTAGCCGTGTAGGC TGGAGCTGCTTC	TCATAACAGAGCCAGCAGAAAGAT CAATTCACCAAGTTCGCATATGAA TATCCTCCTAG
cstA	AATGTAACATCTCTCTGGAACACC CAAACGGACAACAACTGTGTAGG CTGGAGCTGCTTC	CCCTCTCCTTATTCTGGAGAGGGC TATTGATGTAAAAAGACATATGAAT ATCCTCCTTAG
hcr	AAGAAGACATGAAGCAATTG CTGAGCGCGTAAGGAGGTCA GTGTAGGCTGGAGCTGCTTC	GCGCTGACGCTTACCGGGCC TACGATGGAACGTTTACCGA CATATGAATATCCTCCTTAG
hmpA	ATGCTTGACGCACAAACCATCGCT ACAGTAAAGGCCACCAGTGTAGG CTGGAGCTGCTTC	TTACAGCACTTTATGCGGGCCGAA GCATTCGTAATGAATGCATATGAA TATCCTCCTTAG
metE	TCGAAACGCGTCTGGATACGGAA GTTAAAAGCTGGTTTGCGTGTAGG CTGGAGCTGCTTC	TATTTCGCCTGACGCAGATTGTGC GCCGCTTTGACCATGCATATGAAT ATCCTCCTTAG
metH	GTGAGCAGCAAAGTTGAACAACTG CGTGCGCAGTTAAATGGTGTAGG CTGGAGCTGCTTC	TCAGTCCGCATCGTAACCCAGGTT CGGCGCGAGCCACCGCCATATGA ATATCCTCCTTAG
metR	ATGATCGAAATTAAACATCTGAAA ACACTCCAGGCATTGCGTGTAGG CTGGAGCTGCTTC	TTACAGGCGCGGCTGTGATCCTG GCTTCGCTGTGGGTGCACATATGA ATATCCTCCTTAG
nsrR	GTGCAGTTAACGAGTTTCACCGAT TACGGCTTACGTGCGCGTGTAGG CTGGAGCTGCTTC	TCACTCCACCAGTAATAATTTATAA AGCGGTTGATTCTCTCATATGAAT ATCCTCCTTAG
pocR	AATATCTGAGCTATTCAATTTCACG CCGTTTTGTCAGTTGGTGTAGGCT GGAGCTGCTTC	AAAAGACTATCAAAAATCGGCAAT AGCAAAATATTGCTATCATATGAAT ATCCTCCTTAG
STM1273	CTTATGATTGGGTATTAAAAA CCAGAAGAGTCTTATAATCGT GTAGGCTGGAGCTGCTTC	ATCTATCCCATGTCGAATTCA TGGTCTTTTTTCAGCCGCGCA TATGAATATCCTCCTTAG
STM1808	TTAAATACATCTTTTAATCACCACA TCAGGGAGATGTCTTGTGTAGGCT GGAGCTGCTTC	CTGGGCGGGACGCCGCCCAGTG GTGACTGGTTTACCGATGCATATG AATATCCTCCTTAG
yeaR	TGAGATTATCGCTGAGTAACCTGC GTGAAGAGGGAAGCAAGTGTAGG CTGGAGCTGCTTC	TTATTCGTGACCGTGACGGTATAA GTAGCTTTTCCCATTACATATGAAT ATCCTCCTTAG

Table 4 **Primers used for mutant confirmation**

	Forward Primer	Reverse Primer
pkD3	-	TTATACGCAAGGCGACAAGG
pkD4	-	CAGTCATAGCCGAATAGCCT
cobS	ACTGGTGACAAATGAGGTGG	CGTGCGCAGCAACATATTGA
cstA	CACCCAAACGGACAACAACT	GGGCTATTGATGTAAAAAGA
hcr	CACCGGGCCGACCGCGCCGG	AAGTTTAGTTGAAGATGACG
hmpA	TTAATGCTATCGCGGCCTAC	ATACTGCCCGGGACGGTATT
metE	CGGAACTGCATCAACGTCTG	CCCGTCAAAGGGTAGCGCCG
metH	GAAAGAGATGGAGCGTCAGG	GGACCGCTGTAGTTCTGCTC
metR	ACTCTAAATAGTTCGGCTT	TCATGAATAACATGCATGTT
nsrR	GTGTCGGGTAAGCCATTACG	TTTCTGCTTCGCGTTCCTGG
pocR	GTCAATATTCATGGTGATGA	ACTATCAAAAATCGGCAATA
STM1273	GCGCTGTTTATTTACATCAG	CGAGTGGTTGAGTTTATAACC
STM1808	GATACACACGCTCCTTCGGG	AACGATGAAAGGGCTTGAGG
yeaR	AAAATACTGTTTTTGGAGCG	GGTACCAGCAACGTCATCGG

Table 5 **PCR components.**

	Volume for 25 µl total reaction (µl)	Volume for 50 µl total reaction
BIOMIX (Bioline) 2x	12.5	25
Forward Primer (1 µM)	1	1
Reverse Primer (1 µM)	1	1
DNA	5	5
dH_2O (Sigma)	5.5	18

Table 6 **PCR program**

	Knockout construction		Mutant confirmation	
	Temperature (°C)	Time (Minutes)	Temperature (°C)	Time (Minutes)
1: Initial denaturation	95	2	95	2
2: Denaturation	95	2	95	2
3: Annealing	45-60	1	50	1
4: Elongation	72	2	72	2
5: Repeats (Steps 2-4)	35x		25x	
6: Final elongation	72	10	72	10

2.7 Gel electrophoresis

Gel electrophoresis was used both to check quantity and quality of DNA during cloning and to confirm correct mutant construction. Either 5 µL of PCR product or 5 µL purified DNA product or plasmid mixed with 1 µL loading dye was used. Gels were 1% (w/v) agarose gel containing 0.004% (v/v) Ethidium Bromide, made and run in TBE buffer (Thermo Fisher). The gel is run at 100 V for 45 minutes. For size comparison, a 1 kilobase (kb) Hyperladder (Bioline) is run as a marker. Imaging of the gel is done under exposure to UV light.

2.8 B_{12} bioassay

The method for measuring vitamin B_{12} was optimised from (Lawrence and Roth 1996). Strains were grown anaerobically in M9 minimal media for 24 hours, without methionine and with the addition of nicotinic acid (2 ng/ml), aspartic acid (50 ng/ml), thiamine (100 µM), leucine (5 µg/ml), cysteine (50 ng/ml), cobalt (II) chloride (5 µM) and 0.2% (v/v) 1,2-propanediol. Cells were harvested by centrifugation for 10 mins (4000 rpm) and pellet was resuspended in lysis buffer (45 mM Tris, 90 mM NaCl, 1% (v/v) SDS) and boiled for 15 minutes. A *ΔmetE* LB O/N was washed 3X in PBS. Minimal M9 agar (3g agar, 160 mL dH2O, 40 mL 5xM9 minimal media) with a *ΔmetE* top agar was prepared. Discs were placed on-top of agar with 10 µL of each strain to be tested. Plates were incubated O/N at 37 °C and the size of bacterial growth measured the following day. A standard curve was generated using samples of known concentration of vitamin B_{12}.

2.9 Measurement of nitrogen cycle intermediates

Headspace gas samples (3 mL) were taken using a 5 mL gas-tight syringe (Hamilton) and stored in 3 mL pre-evacuated screw cap EXETAINER® vials (Labco). N_2O gas samples were analysed by GC through injection of a 50 µL sample into a Clarus 500 gas chromatographer (PerkinElmer) with an electron capture detector and Elite-PLOT Q [DVB Plot column, 30 m × 0.53 mm ID, carrier: N_2, make-up: 95% (v/V) argon/5% (v/v) methane]. Standards of N2O [5, 100, 1,000, 5,000, and 10,000 ppm (Scientific

and Technical Gases)] were used to quantify N_2O levels. Total N_2O amounts were calculated by applying Henry's Law constant for N_2O at 30°C, KH cc of 0.5392.

NO levels in whole Duran bottles (200ml) were measured using the Nitric Oxide Analyser (NOA 280i, General Electric). This method relies on calibration of the NOA with 90 ppm NO gas, and provides a read-out in ppm. For NO measurements, the entire headspace of the bottle was measured, this is required for the analyser to reach a steady level for accurate measurement of the NO concentration. Separate bottles were therefore set up and sacrificed at each timepoint.

1 mL liquid aliquots were sampled for each culture using a needle and syringe. The optical density (λ600 nm) was measured using a SpectraMax M5 spectrophotometer. The sample was centrifuged at 16,000 g for 5 minutes to pellet residual cells and the supernatant stored at -20°C. NO3- and NO2- concentration was measured using NOA, with 20 µL of thawed liquid sample being injected into a purge vessel. The purge vessel contained 3 mL of either (NaI, 1% w/v in acetic acid) for analysis of NO_2^- or (VCl3, 1% HCl) for NO_3^-, and was connected to a chemiluminescence detector. The reducing agent continuously experienced agitation by N_2 in order to transport NO through the NO analyser and to maintain an anaerobic environment.

2.10 Western Blot

WT, *ΔmetE* and *ΔmetEΔpocR* bacteria were grown as anaerobic cultures in 200 mL 1X M9 as described above for 17h and 48h and an aerobic WT culture was grown for 24h in 50 mL 1X M9. A 6 mL aliquot was taken from each culture and centrifuged for 10 mins (4000 rpm). Pellets were resuspended in a mixture of 50 µL B-mercaptoethanol and 950 µL Laemmli sample buffer. Solution was boiled for 5 mins and centrifuged (20,000 x g) for 2 mins. Proteins were separated on 12% (v/v) denaturing gels (SDS-PAGE) at 180 V for 90 mins. One gel was stained with instant blue protein stain (Expedeon), incubated for 1h and de-stained in H2O O/N.

For MetE detection, a nitrocellulose membrane was soaked in methanol for 1 min and then soaked with blotting paper in transfer buffer (48 mM Tris, 39 mM glycine, 0.04% (v/v) SDS) for 5 mins. Electro-transfer occurred for 1 h at 10 V onto membrane. Membrane was blocked with 5% (w/v) milk powder in 1X Tris-buffered saline with Tween 20 (TBST) buffer (20 mM Tris, 150 mM NaCl and 0.1% (v/v) Tween 20) and

incubated O/N at 4 °C. The membrane was incubated for 1h in 1:10,000 anti-MetE rabbit polyclonal antibody (44,45) diluted in 1% (w/v) milk powder in 1X TBST. The membrane was washed twice with 1X TBST for 1 min with agitation then incubated for 1h in 1:10,000 horseradish peroxidase-conjugated anti-rabbit IgG (Pierce, Rockford, IL) diluted in 1% (w/v) milk powder in 1X TBST. The membrane was washed 3X in 1X TBST and then incubated in 1X TBST for 2h both with agitation. Blot was visualised and protein detected using a lumino-based chemiluminescent detection system (Qiagen).

2.11 Gentamicin protection assay

RAW 264.7 cells were maintained in DMEM with 10% foetal bovine serum (FBS) and 2mM L-glutamine in a 37°C, 5% CO_2 incubator. Bacterial strains were freshly cultured on agar plates and the dose added at a multiplicity of infection of 10 to 1 cells which were seeded 48hrs prior to infection. IFN-λ (1000U) was added 24hrs prior to infection, L-NAME (1 mM) was added with the dose and at the same concentration in maintenance media. Strains were left to infect for 1 hr prior to treatment with gentamycin (100 μg ml^{-1}) for a further hour and either lysed or incubated for a further 22hrs with 10 μg ml^{-1} gentamycin. Prior to lysis with 1% Triton X-100 in H_2O, cells were washed twice with PBS and then plated on LB agar and cfu ml^{-1} counts taken.

2.12 MIC assay

Growth was conducted in 96 well plates in SpectraMax M5 plate reader, with culture volumes of 100 uL. Starter cultures were grown overnight in relevant media/conditions, the inoculum was added at a final concentration of 1/300 McFarland standard (Moore and Kaplan 1992). Starting concentrations were as follows, potassium tellurite (20 mM), potassium selenite (4.87 mM) and hydrogen peroxide (5 μM); subsequent concentrations were achieved by serial dilution.

2.13 Statistics

Results for multiple groups were analysed using GraphPad prism v.6 software by ANOVA or student t-test as stated. Significance for all tests taken as $p \leq 0.05$.

3. A link between tellurite, nitric oxide and virulence in *Salmonella* Typhimurium: the role of TehB, STM1808 and YeaR.

3.1 Introduction

As described in the introduction, nitric oxide detoxification is of key importance to the survival of *Salmonella* inside macrophages. This chapter will focus on the characterisation of three proteins, TehB, STM1808 and YeaR, all annotated as tellurite resistance proteins, namely in their roles in both tellurite and nitric oxide protection.

In a prior study in the Rowley group, a microarray was conducted in order to identify proteins involved in anaerobic nitric oxide protection using RNA extracted from *S*. Typhimurium strain SL1344 cultured anaerobically on glucose medium and exposed to 40 μm NO (Anke Arkenberg, personal communication). Three genes, *STM1808, yeaR and tehB*, all annotated as tellurite resistance genes, were amongst the genes significantly up-regulated, (37, 11 and 15 fold respectively) post NO addition (Figure 7). The fact that all three genes were up-regulated and share the same predicted function warranted further inquiry into their individual and combined role in NO detoxification and forms the basis of this study.

The three proteins, STM1808, TehB and yeaR have numerous similarities which will be discussed, but most importantly, they are all regulated by the nitric oxide-sensitive repressor NsrR (Karlinsey et al. 2012). NsrR belongs to the Rrf2 family of transcriptional repressors and senses NO specifically at a [2Fe-2S] site (Tucker et al. 2008). The presence of the [Fe-S] clusters makes NsrR highly sensitive to damage by NO so that genes repressed by NsrR, are de-repressed following NO exposure. In addition to the three proteins studied here, the NsrR regulon of *Salmonella* consists of other proteins known to be involved in NO protection, including at least *hmpA*, *ytfE*, *ygbA* and *hcp-hcr* (Gilberthorpe et al. 2007, Bang et al. 2006, Karlinsey et al. 2012).

Tellurite is an oxyanion of tellurium which is highly toxic to a large range of bacteria (Taylor 1999). The cause of tellurite toxicity is not fully understood, however it is thought that tellurite interacts with both cellular thiols and glutathione which leads to its reduction to elemental tellurium (Te^0) and the release of superoxide, the elimination of which results in oxidation of cellular thiols and in turn the shutdown of multiple essential processes (Taylor 1999, Turner et al. 2001, Turner, Weiner and Taylor 1995b)

Across bacteria there are at least five known tellurite resistance (Te^R) determinants, located both on the chromosome and on plasmids, all of which are unrelated at the level of DNA/protein structure and includes *tehAB, klaABC, terZABCDEF, tmp*, *trgAB* and *cysK* (Taylor 1999). Studies looking at these different systems suggest they work in very different ways. *Rhodobacter sphaeroides*, which is resistant to approximately 4 mM tellurite (1000 fold higher than *Salmonella*) contains two of the known tellurite resistance determinants, *trgAB* and immediately downstream, *cysK (*the latter also found in *E. coli* and *Salmonella*). TrgAB encodes a membrane-associated protein while CysK is required for cysteine synthesis (Moore and Kaplan 1992, O'Gara, Gomelsky and Kaplan 1997). The KlaABC system does not require a cysteine metabolic pathway or glutathione whereas TehAB does (Turner et al. 1995b). The Te^R determinant, Tmp*,* identified in the pea blight pathogen *Pseudomonas syringae*, appears to be a thiapurine methytransferase. Similarly, TehAB*,* which is also found in *E. coli* and *Salmonella,* contains motifs associated with SAM-dependent N-methyltransferases (Turner, Weiner and Taylor 1995a). Additionally, TehA has been shown to provide resistance against disinfectants and antiseptics as well as protecting the cells from uncoupling (Turner, Taylor and Weiner 1997, Lohmeier-Vogel, Ung and Turner 2004). Furthermore it is known that TehAB requires a functional electron transport chain and a functional quinone pool in order to provide resistance against tellurite (Turner et al. 1995b).

The fact that a number of these systems require either strong up-regulation or mutations in the genes before Te^R is observed, combined with the knowledge that most bacteria will not encounter tellurite at significant levels, suggests that Te^R may not be the primary function of these proteins (Taylor 1999). However, the existence of several separately evolved proteins with overlapping functions suggests a level of physiological importance. This is even more noticeable in *Salmonella.* Of the three tellurite resistance proteins studied here, TehB, STM1808 and YeaR, there is 46% amino acid identity between STM1808 and YeaR but no clear homology between TehB. One of these genes, STM1808, is not present in the close relative *E. coli* suggesting an increased requirement for the function of Te^R systems in *Salmonella.*

Of the three Te^R proteins in this study, TehB has been the best characterised in *E. coli* studies. It has, however, been reported that TehB in *Salmonella,* which is homologous

to TehB from Haemophilus *influenzae* does not have homology in the domain that functions as an *S*-adenosyl-L-methionine (SAM)-dependent methyltransferase that is considered to be required for tellurite resistance (Karlinsey et al. 2012).

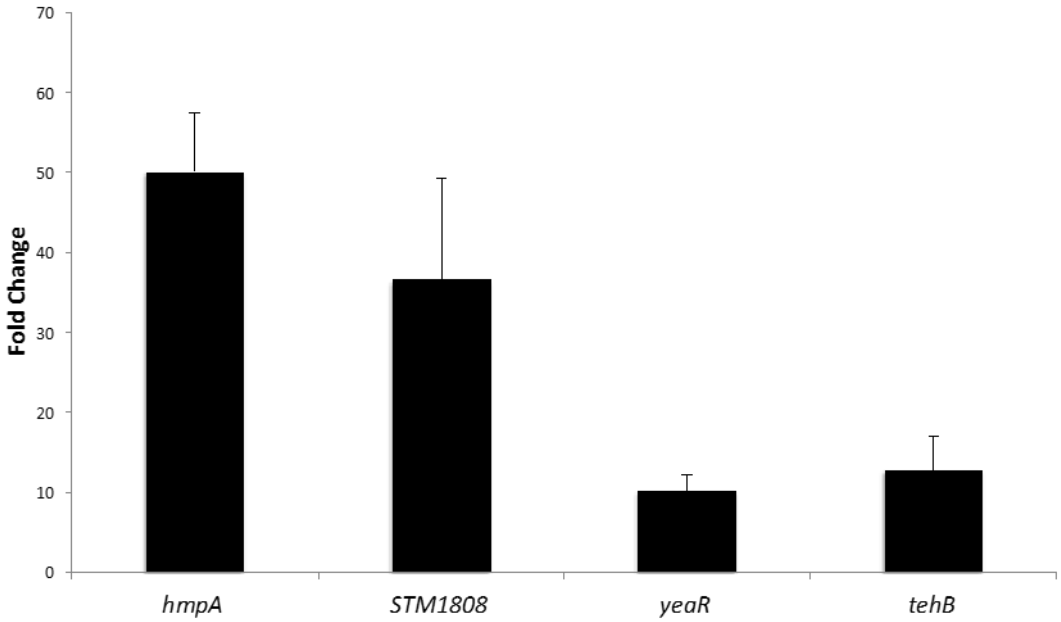


Figure 7 **Tellurite resistance genes are up-regulated by exogenous nitric oxide**. RNA was extracted from anaerobic cultures of *Salmonella* Typhimurium. Fold change in expression between 0 and 10 mins post 40μm NO addition, as determined by microarray analysis, is plotted. Data shown is the mean of 4 biological replicates. Error bars represent standard error. (Data provided by Anke Arkenberg, personal communication)

3.1.1 Aims

This chapter aims to characterise TehB, TehB and STM1808 in relation to their roles in nitric oxide protection by:

- Creating knockout mutants of the three genes, including a triple knockout strain
- Monitoring the survival of the strain in the presence of tellurite, aerobically and anaerobically
- Determining the role the proteins play in NO protection and detoxification, both by monitoring growth and measuring NO levels produced by denitrifying strains
- Investigate the tellurite resistance properties of known nitric oxide detoxification systems
- Assess the survival of the mutant strains in macrophages

3.2 Results

3.2.2 Mutant construction

Knockout mutant constructs have been created for *STM1808, yeaR* and *tehB* using the λ-red method in SL1344 (Datsenko and Wanner 2000) and described in detail in the materials and methods. The single mutants have the pkD3 or pkD4 antibiotic resistance cassette remaining whereas the *ΔtehB ΔSTM1808 ΔyeaR* triple mutant has the cassette flipped out for the *tehB* and *yeaR* mutations but remaining for *STM1808*. The cassettes were removed using pCP20. Constructs were confirmed using colony PCR using both external binding primers and those that bind to the cassette combined with an external primer.

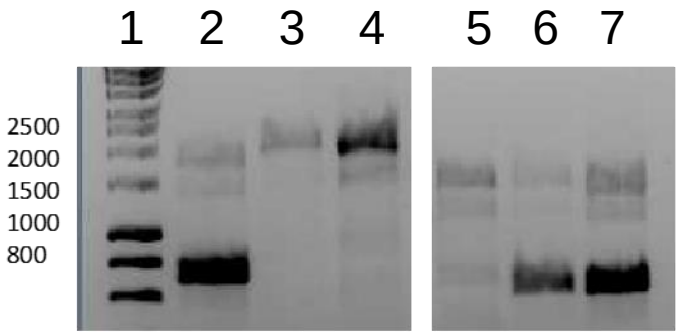


Figure 8 **PCR Verification of STM1808 mutant.** PCR run as detailed in Methods with an annealing temperature of 50ºC and resulting products run on an electrophoresis gel. Lane 1: Hyperladder . Lanes 2-4: External *STM1808* primers. Lane 2: WT, expected size 660; Lane 3:*STM1808* Kan 1, expected size 2078; Lane 4:*STM1808* Kan 1, expected size 2078. Lanes 5-7: External STM18*08* forward primer, internal pkD4 reverse primer. Lane 5: WT, expected no band; Lane 6: *STM1808* Kan 1, expected size 789 Lane 7:*STM1808* Kan 1, expected size 789.

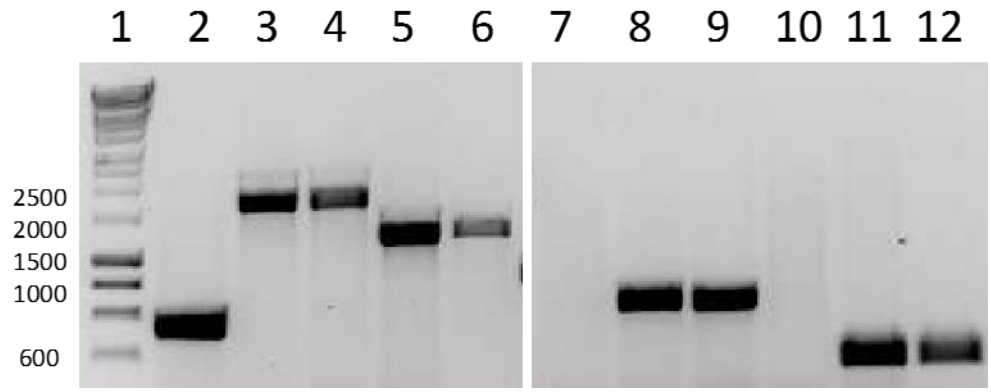


Figure 9 **PCR Verification of *yeaR* mutant.** PCR run as detailed in Methods with an annealing temperature of 50ºC and resulting products run on an electrophoresis gel. Lane 1: Hyperladder . Lanes 2-6: External *yeaR* primers. Lane 2: WT, expected size 540; Lane 3: *yeaR* Kan 1, expected size 1817; Lane 4: *yeaR* Kan 3, expected size 1817; Lane 5: *yeaR* Cm 2, expected size 1417; Lane 6: *yeaR* Cm 5, expected size 1417. Lanes 7-9: External *yeaR* forward primer, internal pkD4 reverse primer. Lane 7: WT, expected no band; Lane 8: *yeaR* Kan 1, expected size 609; Lane 9: *yeaR* Kan 3, expected size 609; Lanes 10-12: External *yeaR* forward primer, internal pkD3 reverse primer. Lane 10: WT, expected no band; Lane 11: *yeaR* Cm 2, expected size 325; Lane 12: *yeaR* Cm 5, expected size 325.

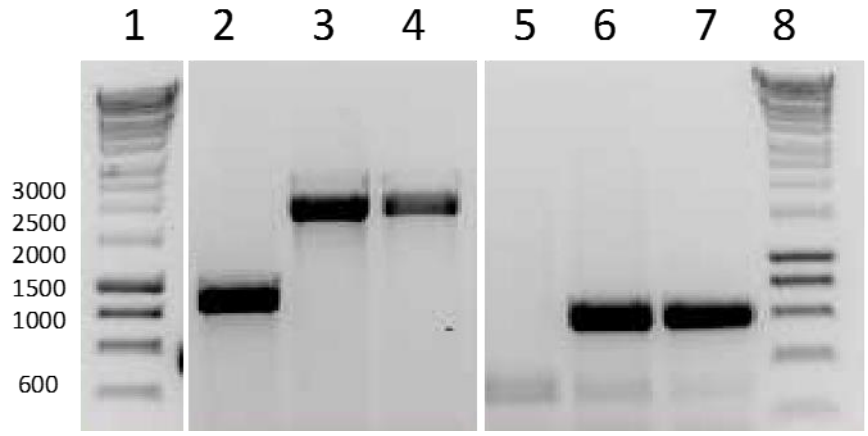


Figure 10 **PCR Verification of *tehB* mutant.** PCR run as detailed in Methods with an annealing temperature of 50ºC and resulting products run on an electrophoresis gel. Lanes 1 and 8: Hyperladder . Lanes 2-6: External *tehB* primers. Lane 2: WT, expected size 800; Lane 3: *tehB* Kan 2, expected size 1903; Lane 4: *tehB* Kan 4, expected size 1903; Lanes 5-7: External year forward primer, internal pkD4 reverse primer. Lane 5: WT, expected no band; Lane 6: *tehB* Kan 2, expected size 629; Lane 7: *tehB* Kan 4, expected size 629

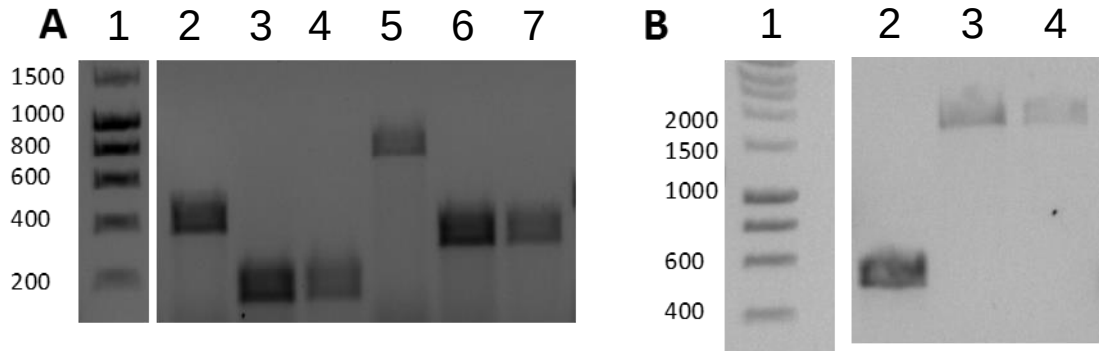


Figure 11: **PCR Verification of *yeaR tehB STM1808 triple* mutant.** PCR run as detailed in Methods with an annealing temperature of 50ºC and resulting products run on an electrophoresis gel. A: Lane 1: Hyperladder. Lanes 2-4: External *yeaR* primers. Lane 2: WT, expected size 540. Lane 3: *yeaR tehB STM1808* 1, expected size 177; Lane 4: *yeaR tehB STM1808* 6, expected size 177. Lanes 5-7: External *tehB* primers: Lane 5: WT, expected size 800. Lane 6: *yeaR tehB STM1808* 1, expected size 403; Lane 7: *yeaR tehB STM1808* 6, expected size 403. B: Lane 1: Hyperladder. Lanes 2-4: External *STM1808* primers. Lane 2: WT, expected size 720. Lane 3: *yeaR tehB STM1808* 1, expected size 1817; Lane 4: *yeaR tehB STM1808* 6, expected size 1817.

3.2.3 There is no general growth defect for the Te^R mutants cultured aerobically in LB

To ascertain whether the mutations in *yeaR, tehB, STM1808* had any impact on the ability of *Salmonella* to grow aerobically in rich media all mutants were grown in LB. Figure 12 shows that all strains grew like WT and therefore we were happy to continue with further phenotyping experiments.

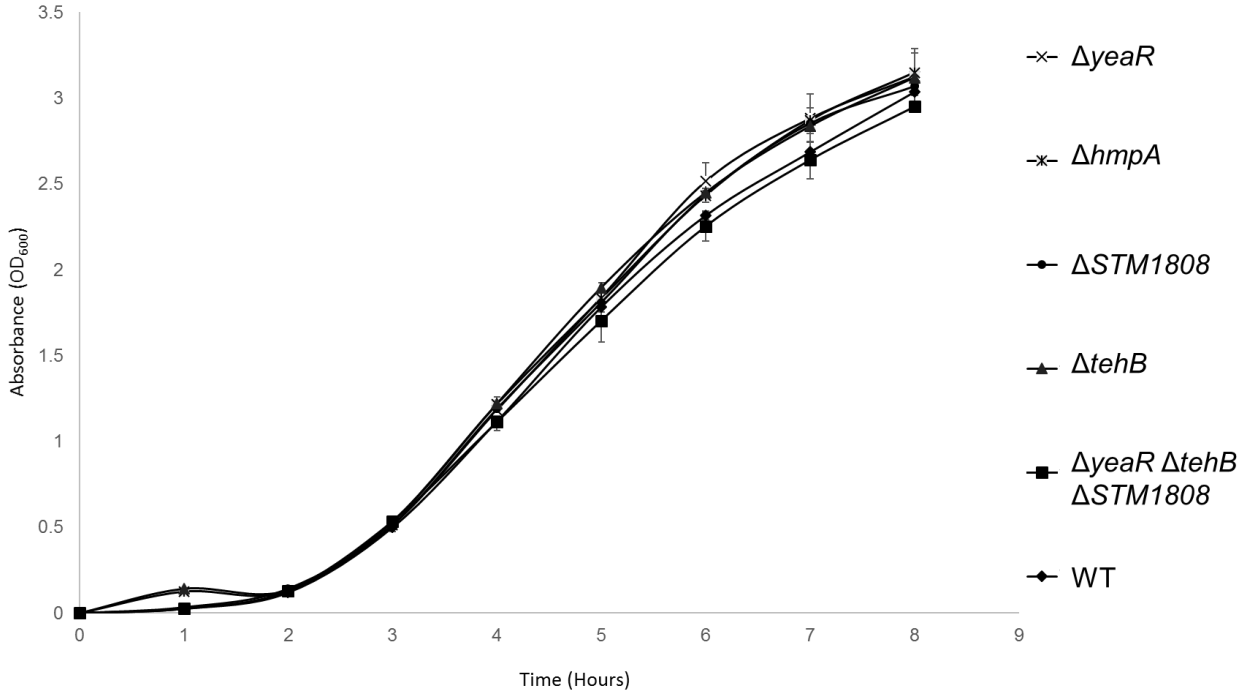


Figure 12 **The four single Te^R mutants and triple Te^R mutant have no general growth defect in LB aerobically.** Strains were grown in 50ml at 200rpm.Samples taken every hour and optical density read at 600nm. 3 repeats were used and SE is shown.

3.2.4 TehB, STM1808 and TehB all contribute to tellurite resistance in *Salmonella*

Although referred to as putative tellurite resistance proteins on the basis of their genome annotation (and contain a conserved domain, DUF1971, linked with tellurite resistance) (NCBI.com, 2017) there is currently no experimental evidence, confirming STM1808 and TehB as tellurite resistance proteins in *Salmonella* Typhimurium, indeed it has been proposed that there is no role of these proteins in tellurite resistance (Karlinsey et al. 2012). To determine whether they have any role in tellurite resistance in *Salmonella* Typhimurium, *ΔyeaR, ΔSTM1808, ΔtehB* and *ΔtehB ΔSTM1808 ΔyeaR* were tested for their ability to grow, aerobically and anaerobically, in the presence of 5 μM potassium tellurite (Figure 13).

This concentration was chosen due to the fact that the minimum inhibitory concentration (MIC) of tellurite for *E. coli* is between 3.94-7.88 μM (Taylor 1999). In both conditions the addition of tellurite had a clear toxic effect on WT *Salmonella,* with a ~70% reduction in OD at 24 hours aerobically, and ~50% reduction under anaerobic conditions. We, however, focused on how the mutant strains grew in comparison to the WT with the addition of tellurite. Aerobically there is a clear lag in growth for both *ΔSTM1808* and *ΔyeaR,* which is less pronounced anaerobically, especially for *ΔyeaR.* The growth defect seen for *ΔtehB* both aerobically and anaerobically is much more pronounced (with a similar growth pattern seen for *ΔtehB ΔSTM1808 ΔyeaR)* with almost no growth observed. The data demonstrates an important role for TehB in protecting *Salmonella* against tellurite*,* with STM1808 and TehB also providing a degree of tellurite resistance (Te^R).

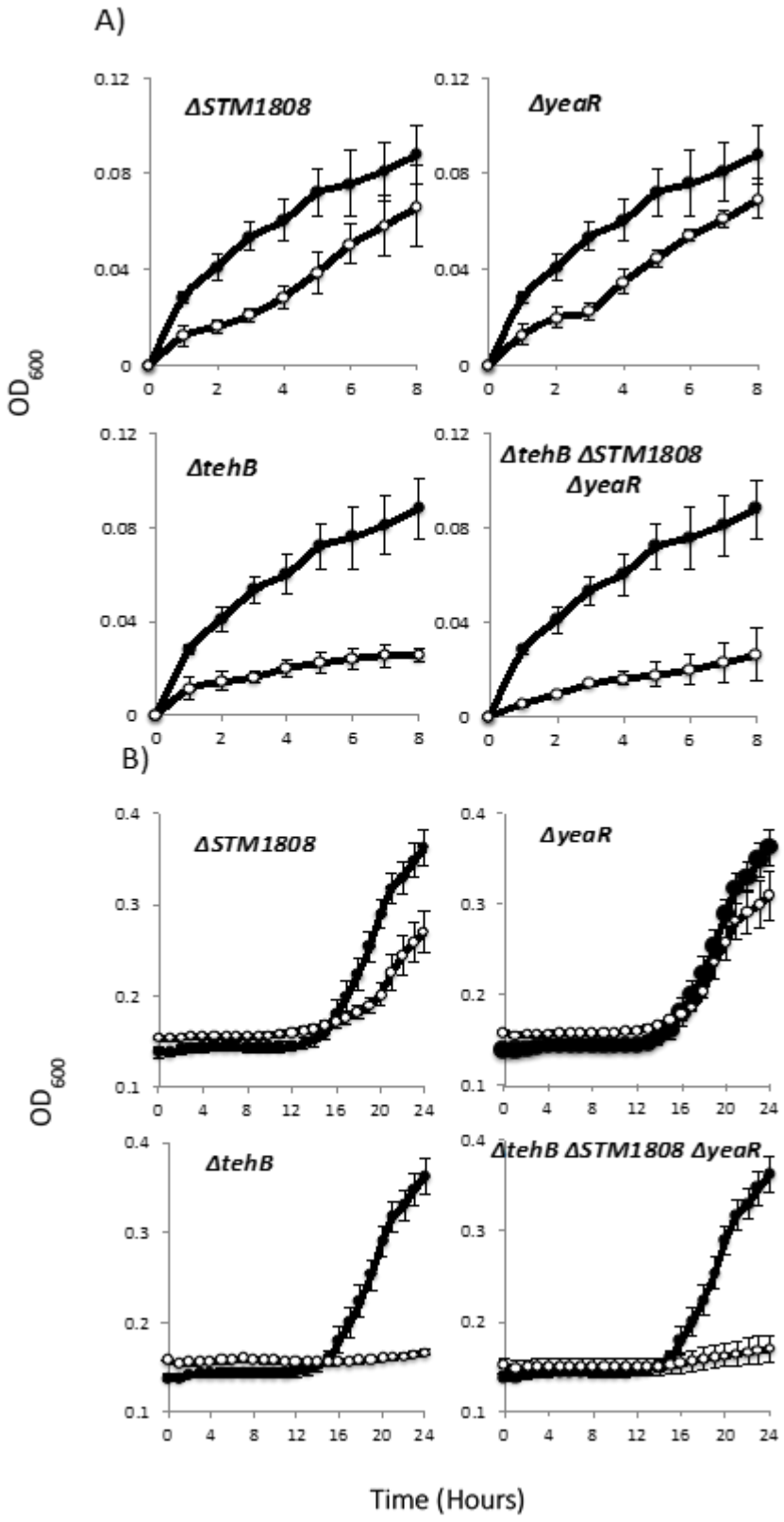


Figure 13 **In *Salmonella* resistance against potassium tellurite requires TehB, STM1808 and YeaR, both aerobically and anaerobically.** Strains were grown aerobically in 50ml LB (A) or anaerobically in 1ml LB (B) with the addition of 5 μM potassium tellurite

3.2.5 Functional overlap between genes required for tellurite resistance in anaerobic protection against nitric oxide

Since expression of the three Te^R genes was induced in the presence of NO the mutant strains were then grown both aerobically and anaerobically in LB in the presence of deta NONOate, an NO donor (Figure 14).

The addition of 5mM deta NONOate to LB has been shown to result in a maximum of 8 μM of NO being released, reducing to 4 μM after 24 hours, we can therefore estimate that the addition of 10mM deta NONOate would result in a maximum of ≈16 μM of NO (Henard and Vázquez-Torres 2012). We used *ΔhmpA* as a negative control and *ΔnsrR* as a positive control. Consistent with previous studies (Gilberthorpe et al. 2007) there is a growth defect for *ΔhmpA* both aerobically and anaerobically when exposed to an NO donor and an increased rate of growth for *ΔnsrR* aerobically. The increase in growth rate for *ΔnsrR* is depressed anaerobically, presumably due to the anaerobic importance of NrfA and NorV which are controlled by additional regulators. Aerobically, in the presence of NO, there is no growth defect for the single mutants, *ΔyeaR, ΔtehB, ΔSTM1808,* mutants nor for the triple Te^R mutant strain *ΔtehB ΔSTM1808 ΔyeaR.* Anaerobically there is no difference in growth between the WT and the single Te^R mutants in the presence of the NO donor, however, in the triple mutant strain *ΔtehB ΔSTM1808 ΔyeaR,* a clear reduction in growth rate and final OD can be seen.

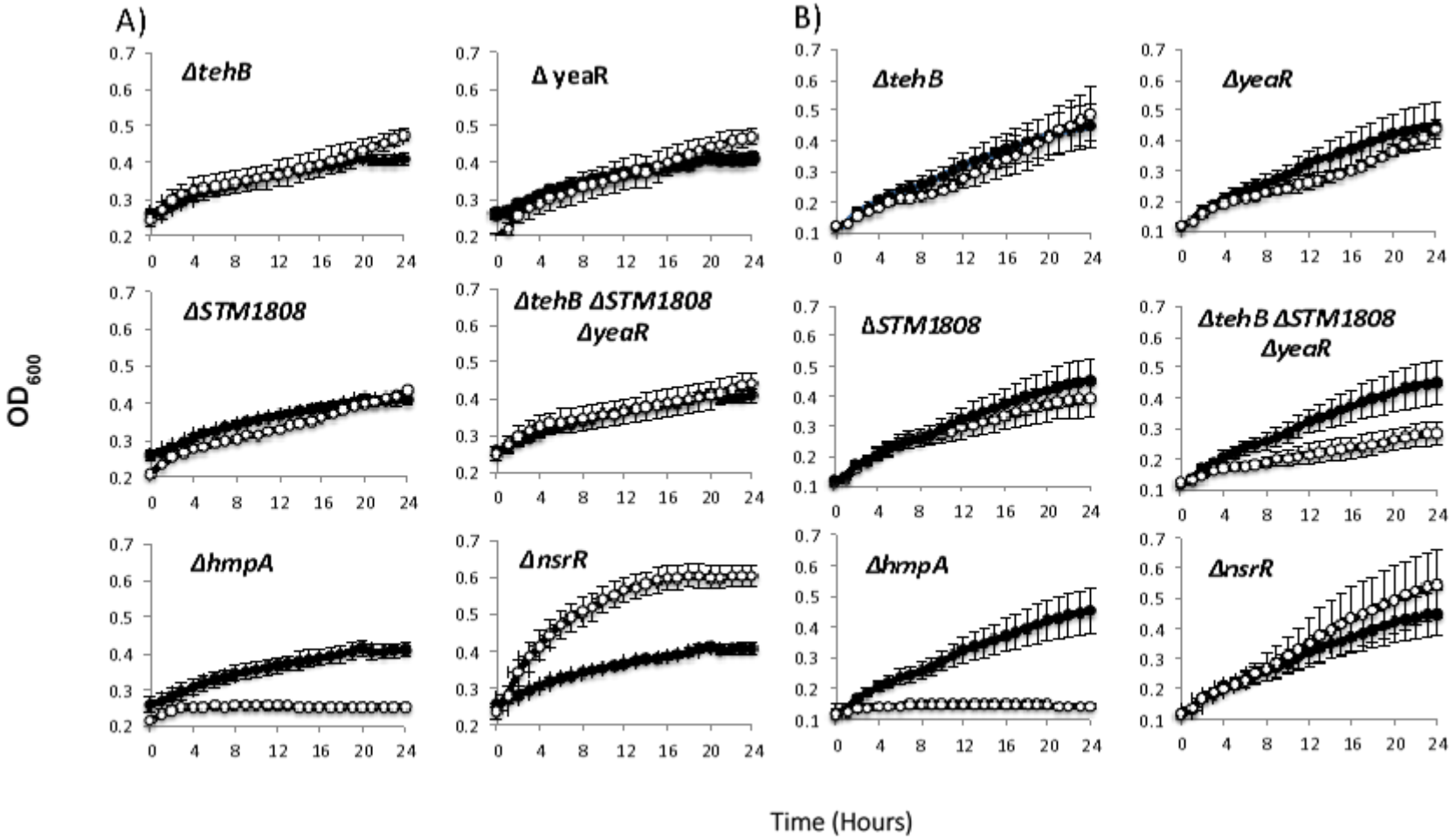


Figure 14 **Functional overlap between TehB, STM1808 and TehB in protection against nitric oxide only during anaerobic growth.** Strains were grown aerobically (A) or anaerobically (B) in 1ml LB with the addition of 10mM Deta NONOate. Each panel shows the WT (black circles) and the indicated mutant strain (white circles). All strains have a minimal of 5 repeats, and standard error is shown.

3.2.6 TehB H32 and H82 are not important for tellurite protection but are important for nitric oxide protection

It has been previously shown in *Salmonella* that two histidine residues which are conserved in the DUF1971 domain are important for the nitric oxide resistance of STM1808 (Karlinsey et al. 2012). Numerous histidine residues were individually mutated to alanines and both single mutants behaved as the total STM1808 knockout strain. Further work then concluded that H82 is an important residue for zinc binding (Karlinsey et al. 2012).

Since YeaR also possesses the DUF1971 domain with these same two conserved histidine residues we hypothesized that these would be equally as important for nitric oxide resistance, and possibly tellurite resistance, as in STM1808 (Figure 15). This conserved domain is not seen in TehB (Figure 16). While both the Karlinsey study and this work saw no growth phenotype for *ΔyeaR* in the presence of nitric oxide, we do see a growth defect in the presence of tellurite. Additionally, the *ΔtehB ΔSTM1808 ΔyeaR* triple mutant strain does have a growth defect in the presence of nitric oxide where a complementation effect might be able to be seen.

Six strains were constructed, the *ΔtehB ΔSTM1808 ΔyeaR* triple mutant was complemented with either WT *yeaR*, *yeaR* with H32 and H82 mutated to alanines, or the empty plasmid, from here referred to as *ΔtehB ΔSTM1808 ΔyeaR* P*yeaR, ΔtehB ΔSTM1808 ΔyeaR* P*yeaRH32AH82A or, ΔtehB ΔSTM1808 ΔyeaR* P*empty,* respectively. The same plasmids were also transformed into the WT strain, with the resulting strains being WT P*yeaR,* WT P*yeaRH32AH82A* and, WT P*empty.*

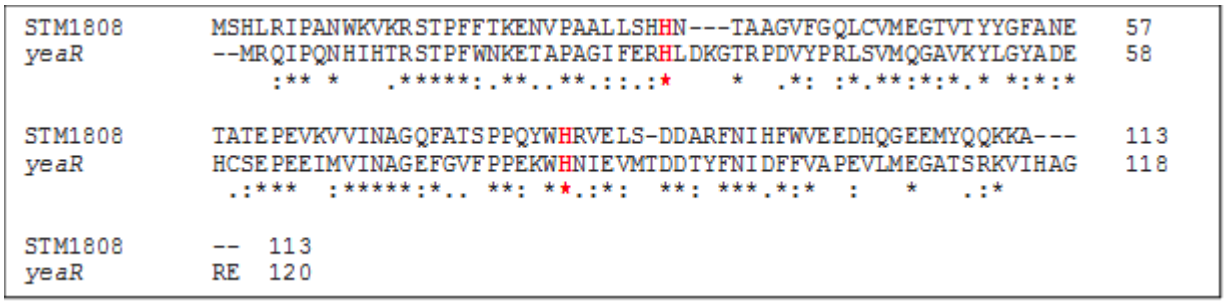


Figure 15 **Alignment of *STM1808* and *yeaR*.** Performed using Clustal W alignment. H32 and H82 are highlighted in red.

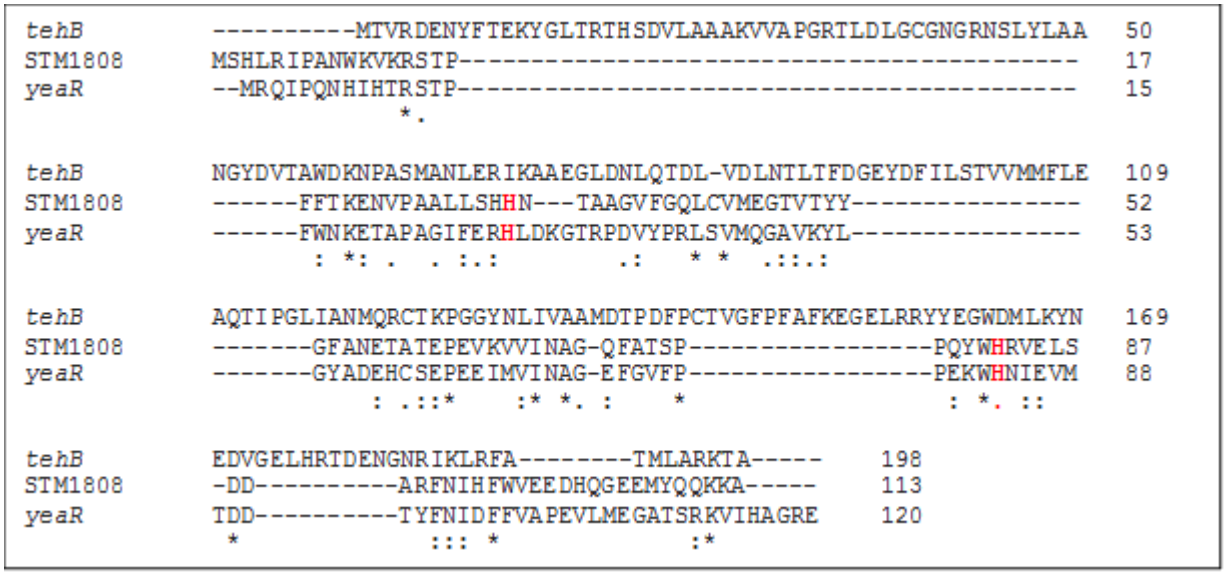


Figure 16 **Alignment of *tehB*, *STM1808* and *yeaR*.** Performed using Clustal W alignment. H32 and H82 are highlighted in red.

All six strains were grown aerobically and anaerobically in LB to confirm no general growth defect or advantages were present (Figure 17). The six strains were then tested for their ability to grow aerobically in LB in the presence of 5 µM potassium tellurite. This was not carried out anaerobically as no growth defect is seen for *ΔyeaR* anaerobically (Figure 13).

The TehB complemented strain, as *ΔtehB ΔSTM1808 ΔyeaR* P*yeaR*, showed a mild restoration in growth in comparison to the control strain, *ΔtehB ΔSTM1808 ΔyeaR* P*empty (*Figure 18*).* Both *ΔtehB ΔSTM1808 ΔyeaR* P*yeaR and the* histidine altered complementation strain, *ΔtehB ΔSTM1808 ΔyeaR* P*yeaRH32AH82A* showed very similar levels of growth; therefore suggesting H32 and H82 do not play an important role in the tellurite resistance functions of TehB.

When grown in the presence of deta NONOate, *ΔtehB ΔSTM1808 ΔyeaR* P*yeaR* shows a clear growth restoration in comparison to the control strain, *ΔtehB ΔSTM1808 ΔyeaR* P*empty (*Figure 19*).* However, *ΔtehB ΔSTM1808 ΔyeaR* P*yeaRH32AH82A* grows comparably to the triple mutant with the empty plasmid, therefore suggesting that these two histidine residues are important for anaerobic protection against nitric oxide.

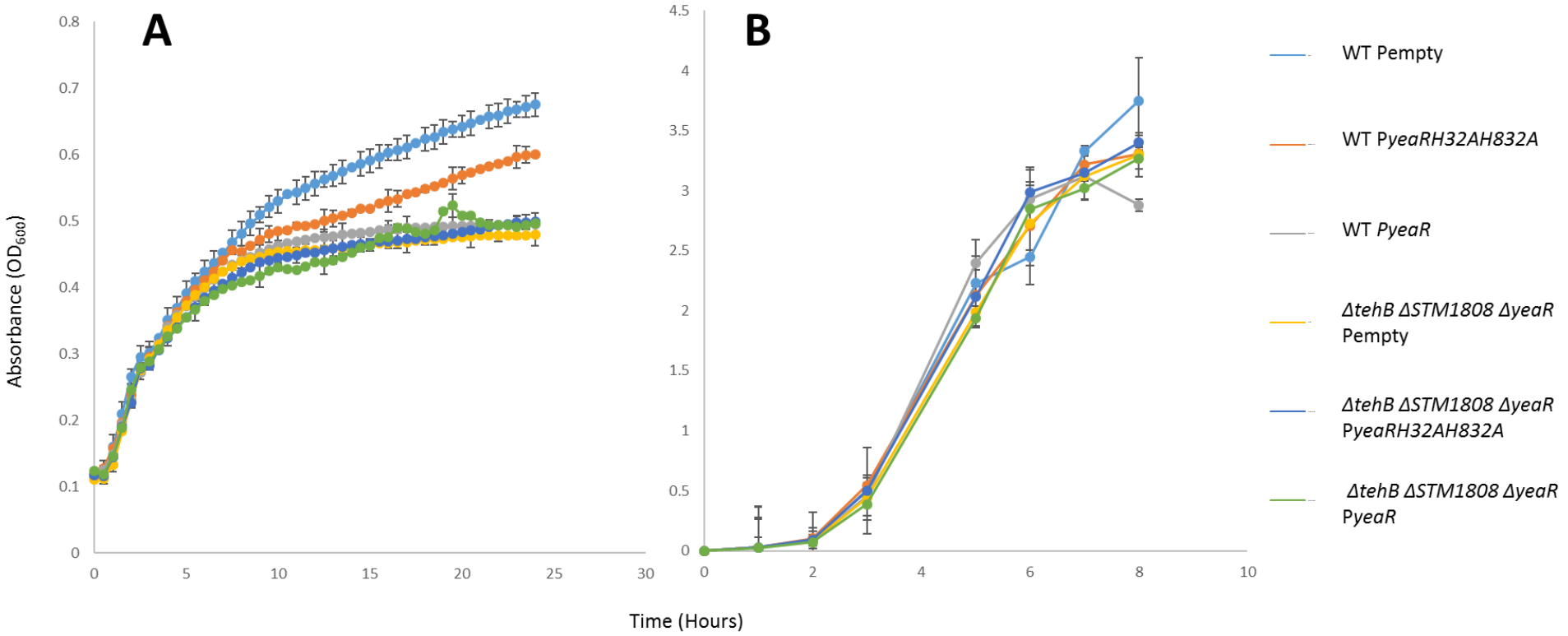


Figure 17 **Complementation strains all grow comparably both aerobically and anaerobically.** Strains were grown anaerobically in 1 ml LB (A) or aerobically in 50 ml LB (B) with the addition of 100 µg/ml ampicillin and 1 mM arabinose

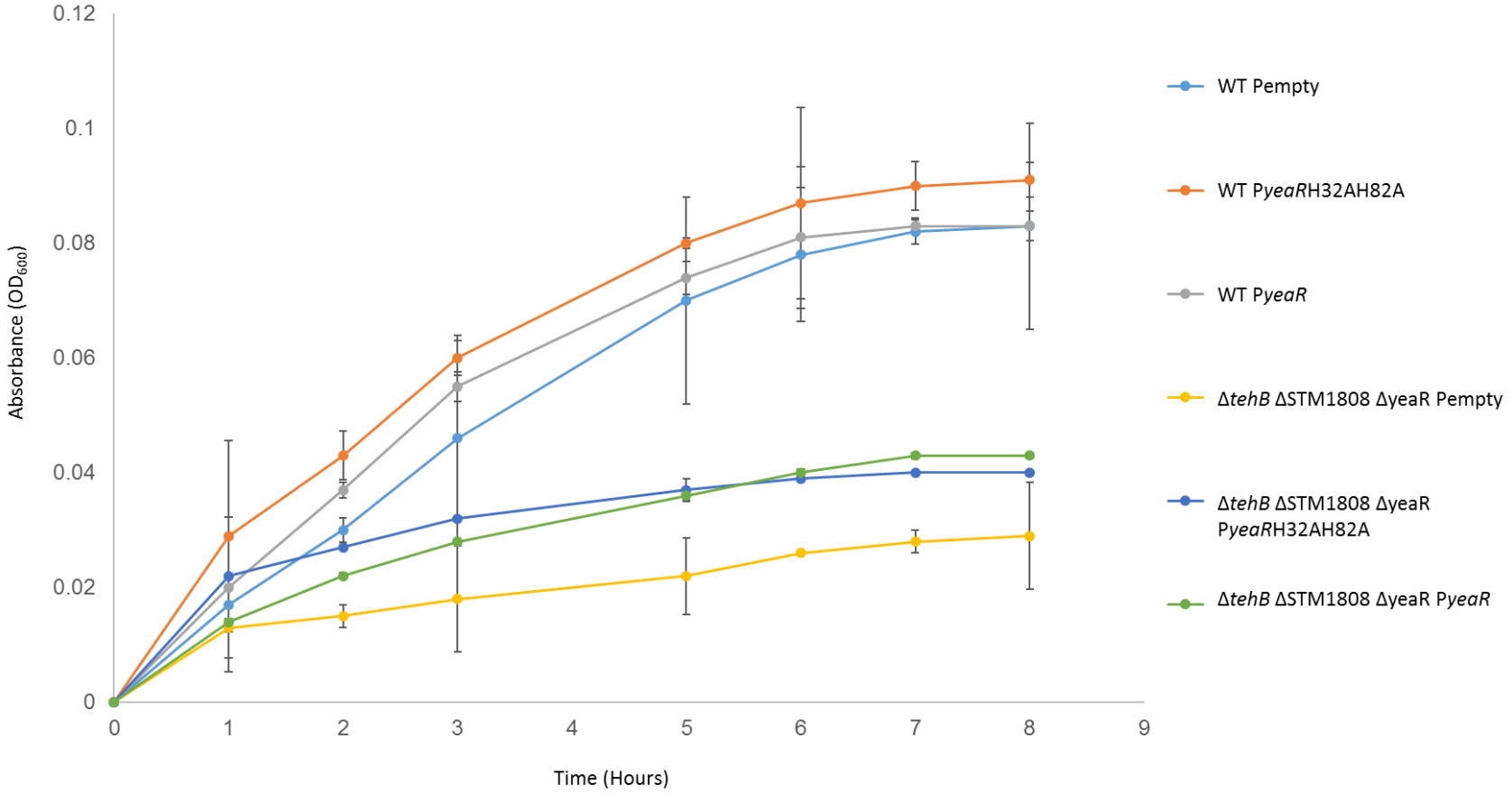


Figure 18 **Complementation with *yeaR* or *yeaR*H32AH82A provides a low level of growth restoration of Δ*tehB* *ΔSTM1808* Δ*yeaR* when grown in the presence of potassium tellurite.** Strains were grown aerobically in 50ml LB with the addition of 5 µM potassium tellurite, 100 µg/ml ampicillin and 1 mM arabinose.

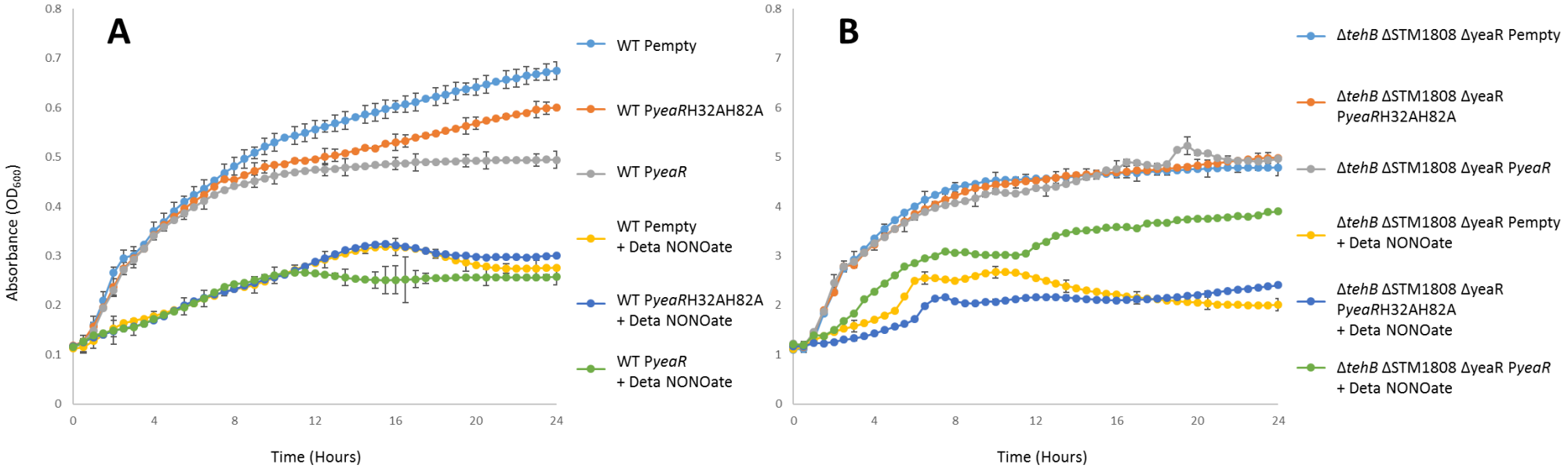


Figure 19 **H32 and H82 are important for year mediated nitric oxide protection anaerobically.** Strains were grown anaerobically in 1 ml LB with the addition of 5mM Deta NONOate, 100 µg/ml ampicillin and 1 mM arabinose.

3.2.7 Nitric oxide accumulates during nitrate respiration in *ΔtehB ΔSTM1808 ΔyeaR* triple mutant

In order to ascertain if the Te^R proteins have a direct role in detoxifying NO we grew the strains anaerobically with nitrate as the alternative electron acceptor and measured production of endogenous NO. Firstly the WT strain was grown and 3 ml samples of headspace gas taken at 4 timepoints over 48 hours *(*Figure 20*).* NO levels increased until 40 hours and then were reduced at 48 hours. We therefore determined to efficiently compare levels of NO between the mutant strains measurements should be taken at 40 hours. In order to obtain more accurate NO levels the entire headspace (≈ 50 ml) was used for future results, the experiment was therefore sacrificed at this timepoint.

Under these conditions (M9 minimal media, anaerobic, 20 mM nitrate) all the strains grew comparably to WT. To establish the validity of our experimental set up, NO released by Δ*hmpA* and Δ*nsrR* was measured and had higher and lower levels of headspace NO respectively, with 1.59 ±0.16 μM and 0.31 ±0.15 μM NO compared to 0.72 ±0.14 μM from the WT *Salmonella* strain. We observed no significant ($p < 0.05$) difference in levels of NO released from any of the single Te^R mutants, although a slight increase can be seen for *ΔtehB* (1.07 ±0.04 μM NO). However, the triple Te^R mutant strain, *ΔtehB ΔSTM1808 ΔyeaR,* releases significantly ($p < 0.05$) higher levels of NO than the WT, with 1.33 ±0.11 μM NO detected, further supporting the functional redundancy between the Te^R proteins in directly detoxifying NO (Figure 21).

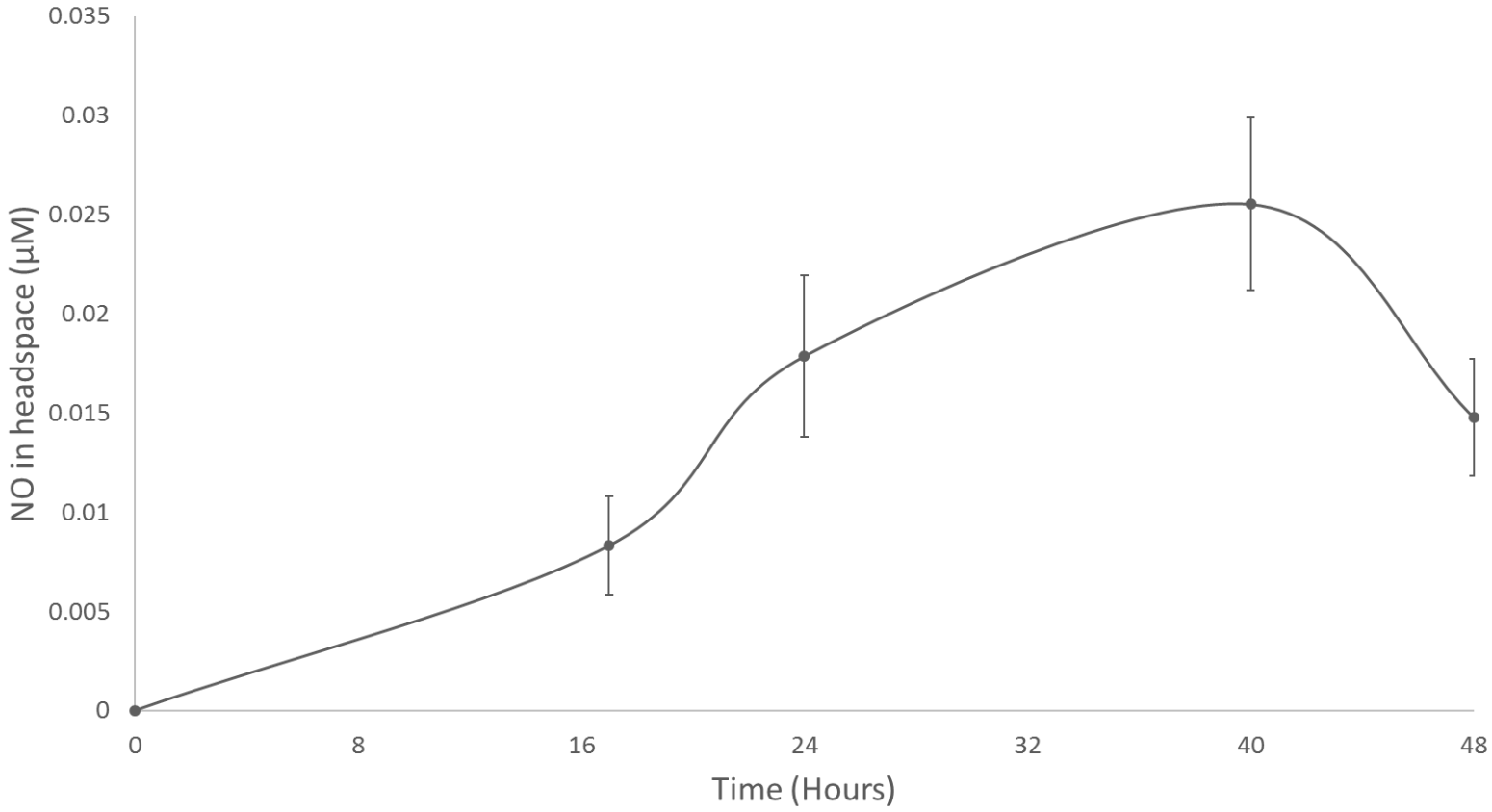


Figure 20 **NO levels peak at 40 hours for WT *Salmonella***. WT was grown in M9 minimal media supplemented with 40mM nitrate and the NO measured from 3ml samples taken from the headspace. 4 repeats were used and SE is shown.

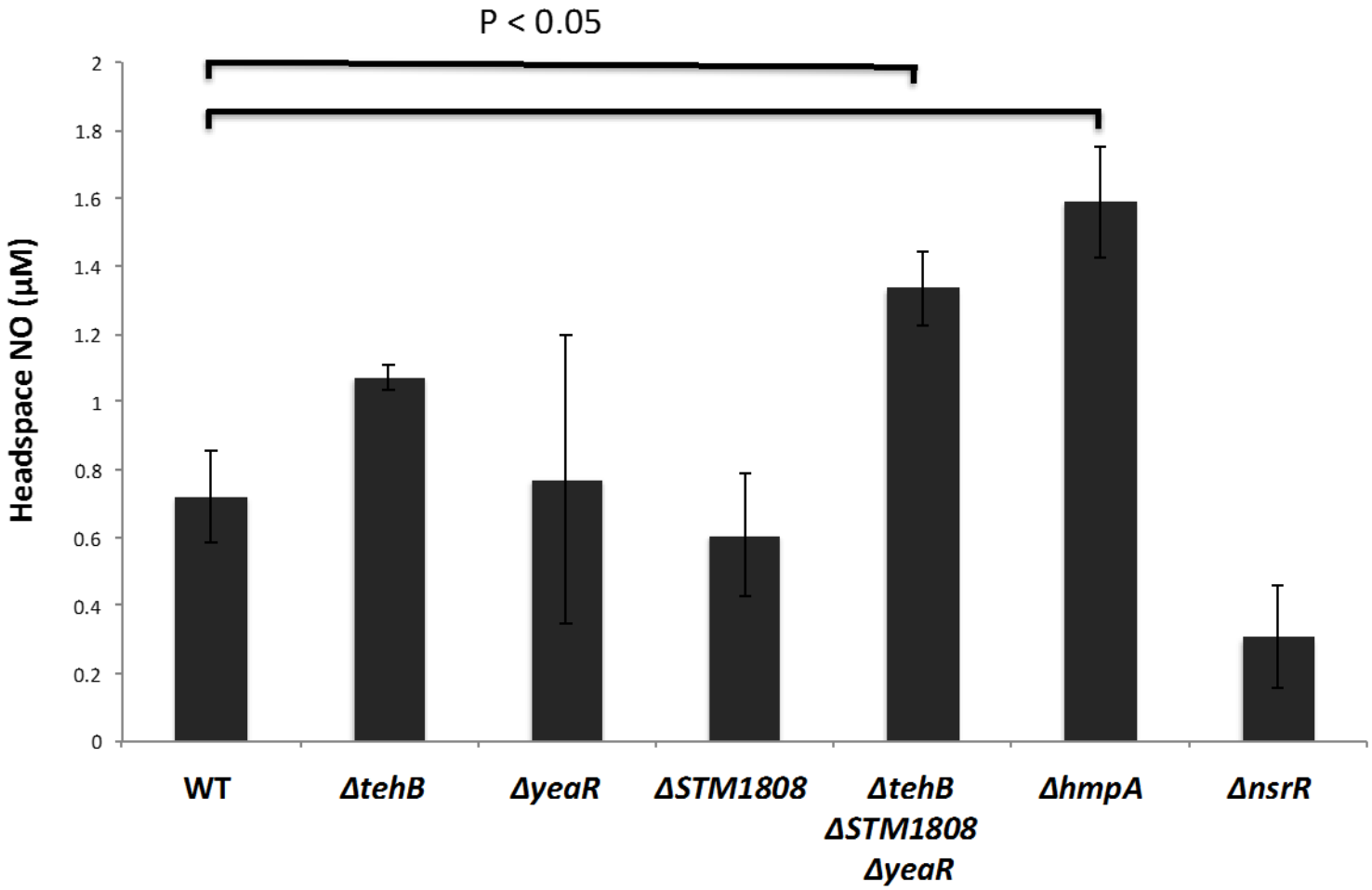


Figure 21 **Relative levels of nitric oxide produced by *Salmonella* mutants**. Cultures were grown anaerobically in M9 minimal media with 20 mM nitrate and 5 mM glycerol with the headspace NO concentration measured after 40 hours of growth. All strains have 3 repeats, and standard error is shown.

3.2.8 Proteins involved in protection against reactive nitrogen species also protect against potassium tellurite

Having demonstrated a role for the Te^R proteins in both NO and tellurite resistance we sought to ascertain whether there is a correlation between tellurite and nitric oxide resistance. To do this four strains, each carrying a mutation in a system implicated in NO protection were exposed to tellurite (Figure 22). Aerobically there was no growth defect for the *ΔytfE, ΔnrfA or ΔnorV* mutants in the presence of tellurite. However, there was a distinct growth defect in the absence of the key NO detoxification protein HmpA. Anaerobically there is a clearer correlation between NO and tellurite resistance with YtfE, NrfA and NorV all exhibiting a degree of protection against tellurite toxicity. HmpA, however, does not appear to have a role in anaerobic tellurite protection. As NsrR is the master regulator of NO detoxification in *Salmonella* the most significant case for support of a correlation between NO and tellurite resistance can be seen with the phenotype of the NsrR mutant. Aerobically the disruption of NsrR, allowing for the up-regulation of the entire NsrR regulon, has a clear benefit to growth in the presence of tellurite in comparison to the WT. After 8 hours the OD_{600} for the *ΔnsrR* mutant had reached ≈0.50 as opposed to ≈0.08 for WT. This enhanced growth phenotype is not apparent in the absence of oxygen, with similar maximal growth for WT and *ΔnsrR.* However, the lag phase is shorter for the *ΔnsrR* mutant (at ~9 hrs) as opposed to ~14 hours for the WT. Additionally, this correlates with a two-fold increase in the aerobic MIC of tellurite for the *ΔnsrR* mutant, 10 μM compared to 5 μM for the WT.

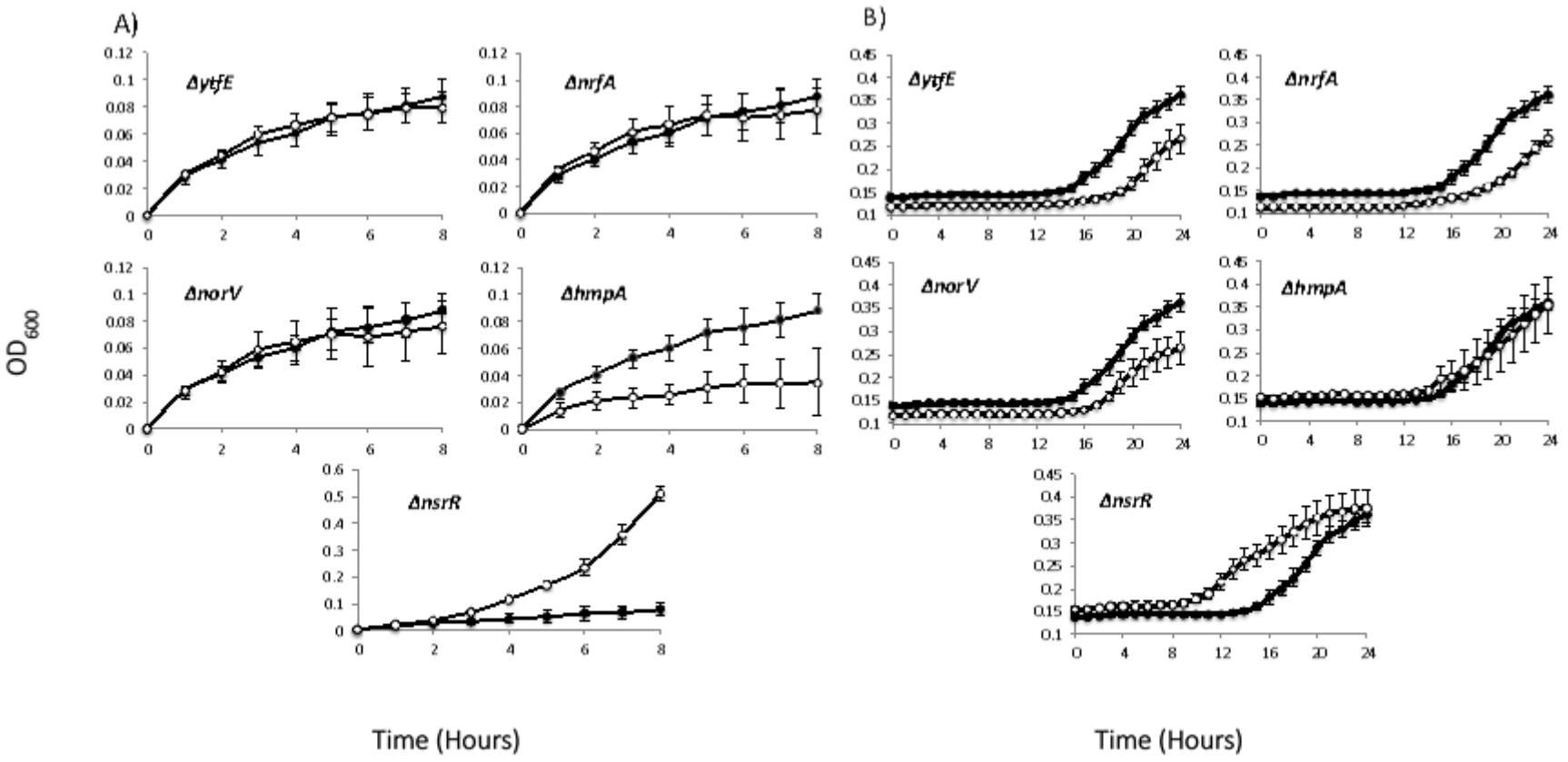


Figure 22 **Genes involved in RNS defense are also involved in resistance against potassium tellurite.** Strains were grown aerobically (A) or anaerobically (B) in 1ml LB with the addition of 5 μM potassium tellurite. Each panel shows the WT (black circles) and the indicated mutant strain (white circles). All strains have a minimal of 5 repeats, and standard error is shown.

3.2.9 MIC of tellurite, selenite and hydrogen peroxide for WT and *ΔtehB ΔSTM1808 ΔyeaR*

The toxicity of tellurite is frequently attributed to the production of ROS (Pérez et al. 2007, Borsetti et al. 2005) and it is also known that at comparable levels selenite produces 4 fold higher levels of ROS than tellurite (Vrionis et al. 2015). One would therefore assume that if the toxicity of tellurite was attributable to the release of ROS that the MIC of selenite would be approximately 4 times lower than that of tellurite. However, we have shown that the MIC of selenite in *Salmonella* is ≈1000 times higher than tellurite (>4.9 mM vs 5.0 μM) suggesting that the toxicity observed with tellurite is not due to production of ROS (Table 7). Importantly, the MICs of H_2O_2 and selenite for the WT and the tellurite hypersensitive strain, *ΔtehB ΔSTM1808 ΔyeaR* are identical at 1.25 μM and >4.87 mM respectively. This suggests that the hypersensitivity to tellurite observed in *ΔtehB ΔSTM1808 ΔyeaR* is therefore not solely due to ROS.

Table 7 **MIC assay.** WT and *ΔtehB ΔSTM1808 ΔyeaR* grown aerobically in 100uL LB to calculate MIC of potassium tellurite (K_2O_3Te), potassium selenite (K_2O_3Se) and hydrogen peroxide (H_2O_2).

	Strain	
Compound	WT	*ΔtehB ΔSTM1808 ΔyeaR*
Tellurite	5.00 μM	2.50 μM
Selenite	> 4.87 mM	> 4.87 mM
Hydrogen Peroxide	1.25 μM	1.25 μM

3.2.10 The *ΔtehB ΔSTM1808 ΔyeaR* triple mutant is severely attenuated in IFN-γ activated macrophages

One of the key host defense mechanisms that challenges *Salmonella* survival in host macrophages is the production of NO via *iNOS.* In macrophages, NO levels can be stimulated by interferon gamma (IFN-γ) or inhibited by addition of NG-nitro-L-arginine methyl ester (L-NAME) upon hydrolysis to NG-nitro-L-arginine (L-NOARG) (Pfeiffer et al. 1996). When L-NAME treated macrophages were infected with either WT or *ΔtehB ΔSTM1808 ΔyeaR* there was no significant difference between the two strains in fold change in bacterial numbers between 2 and 24 hours post infection (pre and post the NO burst). When IFN-γ activated macrophages were infected with either WT or *ΔtehB ΔSTM1808 ΔyeaR* there was significantly ($p< 0.01$) lower survival of *ΔtehB ΔSTM1808 ΔyeaR* in comparison to WT with a 40.8 ±3.87 fold reduction for the triple Te^R strain compared to 10.8 ±3.93 fold for WT (Figure 23).

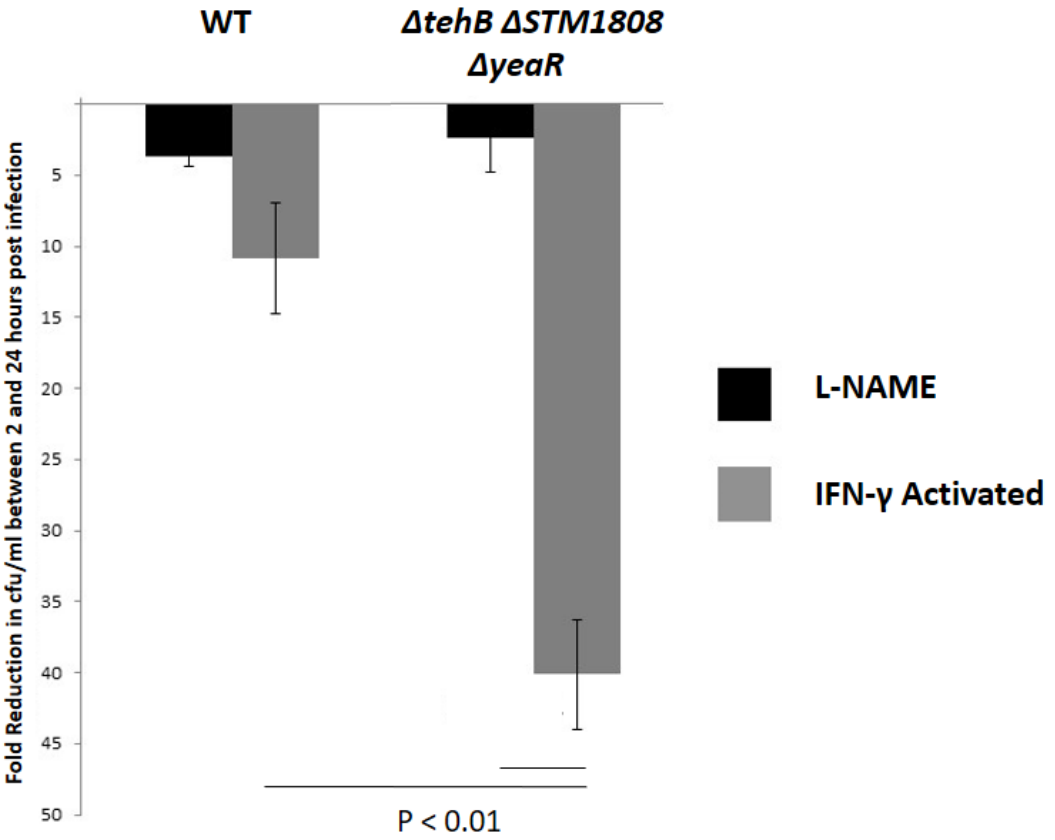


Figure 23 **The Δ*tehB ΔSTM1808* Δ*yeaR* triple mutant is severely attenuated in IFN-γ activated macrophages.** Fold change in cfu/ml between 2 and 24 hours post infection is shown for L-NAME treated (filled) and IFN-y activated (shaded) RAW 264.7 macrophages. 6 repeats were used and SE is shown. A student's t-test was used.

3.3 Discussion:

In this chapter we set out to identify any uncharacterized genes involved in anaerobic NO detoxification by *Salmonella* Typhimurium and in doing so highlighted a connection between NO and tellurite detoxification. We have shown that TehB, YeaR and STM1808 are all involved in tellurite resistance in *Salmonella*, particularly aerobically; and that they provide resistance against NO, which our data suggests they play a role in detoxifying. A *ΔtehB ΔSTM1808 ΔyeaR* triple mutant has also been shown to be severely attenuated in activated macrophages.

Of the three Te^R proteins in this study, TehB has been the best characterised in *E. coli* studies. It has, however, been reported that TehB in *Salmonella,* which is homologous to TehB from *Haemophilus influenzae,* does not have homology in the domain that functions as an *S*-adenosyl-L-methionine (SAM)-dependent methyltransferase that is considered to be required for tellurite resistance (Karlinsey et al. 2012). We however found TehB to be the most important of the three proteins for Te^R in *Salmonella* both aerobically and anaerobically (Figure 14). We have also shown a distinct phenotype for both *ΔyeaR* and *ΔSTM1808* when grown aerobically in the presence of 5 μM potassium tellurite. Anaerobically this phenotype is reduced but is still visible, certainly at least for *ΔSTM1808*. This is in contrast to the suggestion that the methyltransferase domain that is lacking from TehB in *Salmonella* is vital for its role in protecting against tellurite (Karlinsey et al. 2012); and confirms the putative function of all three proteins.

Although we have confirmed here the role of all three proteins, TehB, STM1808 and TehB in Te^R, it remains the case that tellurite is probably not physiologically relevant, at least not at this stage of *Salmonella*'s evolution. Therefore, the fact that *Salmonella* has retained three independent systems for this function does imply that they have another important function and, from the results shown here, we propose this is in NO detoxification. Aerobically there is no growth defect for the mutant strains, including the triple mutant strain in the presence of 10 mM deta NONOate; but anaerobically there is a clear phenotype when all three systems are missing. Previous studies have shown a low level of protection provided by STM1808 against NO added to aerobic cultures, but not a requirement for TehB (Karlinsey et al. 2012). Therefore, we

conclude that there is functional overlap between the three systems for anaerobic NO protection.

In order to ascertain whether this protection is as a direct result of NO detoxification as opposed to responding to the large amount of damage caused by NO in the cell, we grew the mutant strains under denitrifying conditions and measured the levels of NO released (Figure 21). While *ΔtehB* and *ΔyeaR* both accumulated higher levels of NO than the WT this was not significant, however, the triple Te^R showed significantly higher levels of NO release. This confirmed the functional overlap between the three Te^R genes and gives strong indication that they are directly involved in NO detoxification/reduction. It is not clear at this stage the mechanism by which this detoxification occurs and the protein biochemistry of this enzymatic activity warrants further work.

It was noted by Karlinsey *et al*., (2012) that two histidine residues, H32 and H82, in STM1808 are important for the nitric oxide protection exhibited by the protein. These residues are part of the DUF1971 which is conserved within proteins thought to be involved in tellurite resistance. It was highlighted that a mutation of either of these histidine residues in STM1808 was equivalent to knocking out the entire gene when the strain was challenged with nitric oxide. Since STM1808 and YeaR are highly homologous, as shown in Figure 15, with H32 and H82 being conserved, we hypothesized that similarly to in STM1808 these residues would be vital for the role YeaR plays in nitric oxide detoxification. The residues are not conserved within TehB (Figure 16). Since, due to the functional redundancy of TehB, STM1808 and YeaR, we only see a mild phenotype for the single YeaR knockout mutant we therefore complemented the triple Te^R mutant with either wildtype YeaR or YeaR with H32 and H82 mutated to alanines.

These complementation strains were first tested for their ability to grow in the presence of 5 µM potassium tellurite (Figure 18) where there was a 1.5 fold increase in final OD for *ΔtehB ΔSTM1808 ΔyeaR* P*yeaR* compared to *ΔtehB ΔSTM1808 ΔyeaR* P*empty* (0.043 v. 0.029) taking it from 32% of the final OD for WT Pempty to 47%. However, the *ΔtehB ΔSTM1808 ΔyeaR* P*yeaRH32AH82A* strain grew comparably, with a similar final OD of 0.04. In combination these results show that while TehB does play a role in the protection against tellurite in *Salmonella*, these two histidine residues are not important for this function. The role of the histidine residues in tellurite resistance by

STM1808 has not been looked at previously or in this study; this could be something of interest (Karlinsey et al. 2012).

In contrast, there is a role for H32 and H82 in TehB-mediated nitric oxide protection (Figure 19). Complementation of the triple Te^R mutant strain with WT TehB resulted in a restoration of growth, with the final OD reaching 0.390 in comparison to 0.201 for the control strain *ΔtehB ΔSTM1808 ΔyeaR* P*empty* in the presence of deta NONOate and 0.497 in plain LB. Under these conditions however the same was not seen for *ΔtehB ΔSTM1808 ΔyeaR* P*yeaRH32AH82A* with the final OD only slightly higher than the control strain at 0.241. We therefore conclude that H32 and H82 of YeaR are important for nitric oxide, but not tellurite, protection in *Salmonella.* This result has two-fold implications, the first being that there is a clear similarity between the functionality of STM1808 and YeaR in nitric oxide detoxification. In STM1808 these residues have been shown to be important for zinc binding – this could be looked at in YeaR. Secondly, the fact that the two histidine residues are important for one function of YeaR, nitric oxide protection, but not another, tellurite resistance, suggests that the protein is not working in precisely the same way for these two separate functions.

Due to the role of the Te^R proteins in NO detoxification we then tested whether the converse is true, that in *Salmonella* any strain that is sensitive to NO is also sensitive to tellurite; this could give an indication of the cause of toxicity. We demonstrated that the well characterised NO oxygenase/reductase, HmpA, is very important for tellurite resistance aerobically, but not anaerobically. When the NO sensitive repressor, NsrR, is deleted the entire NsrR regulon (consisting of *hcp-hcr, ytfE*, *ygbA, hmp*, *yeaR-yoaG and STM1808)* is upregulated (Karlinsey et al. 2012). In the presence of tellurite *ΔnsrR* grows much better than WT aerobically and, although the phenotype is less pronounced anaerobically, there is still a clear role for the NsrR regulon in tellurite resistance; suggesting that the genes regulated by NsrR play a key role in tellurite resistance as well as in NO resistance. We also tested three further proteins that have been implicated in NO resistance, for their involvement in tellurite resistance. One of these, YtfE (Gilberthorpe et al. 2007) is NsrR regulated, but the other two, NrfA and NorV (Mills et al. 2005) are not. NorV and NrfA are known to be involved in NO resistance anaerobically and we have shown involvement in resistance against tellurite anaerobically. From our data, we conclude that there is a clear correlation between tellurite resistance and NO resistance in *Salmonella*. There is the possibility

that in LB, TeO_3^{-2} leads to the release of NO and therefore the tellurite phenotype, and hence the correlation we are seeing is actually due to NO release post tellurite addition. The mechanism by which this may occur is unknown however there would be a varied mix of nitrogen and oxygen containing molecules present so it is feasible. We however do not believe this to be the case – the phenotypes we see for tellurite and NO are not directly comparable; and moreover we attempted to measure the NO levels from aerobic and anaerobic LB media containing different combinations of bacteria and tellurite and saw no NO release (data not shown). The results from our study on the importance of two histidine residues in YeaR also would not correlate with this hypothesis; the two residues are only seen to be important for protection against NO, if tellurite was simply triggering the release of NO then the residues would be important under both nitric oxide and tellurite stress.

There are indications that tellurite toxicity is as a result, at least in part, of reactive oxygen species (ROS), which are released, when TeO_3^{2-} is reduced to Te. Superoxide dismutase was upregulated in *E. coli* and *Rhodobacter capsulatus* in response to tellurite and, ROS protection mutant strains had increased tellurite sensitivities (Pérez et al. 2007, Borsetti et al. 2005). However, it has been shown that treatment of *E. coli* with selenite results in at least four times as much ROS than tellurite (Vrionis et al. 2015). In our study the MIC of selenite (>4.875 mM) is much higher than that of tellurite (5 μM) (Table 1). This suggests that it is not solely the induction of ROS production that causes the toxic effects of tellurite. We also exposed the tellurite hypersensitive *ΔtehB ΔSTM1808 ΔyeaR* to H_2O_2 and selenite and saw no difference in sensitivity than the WT – indicating that the tellurite sensitivity of this strain is not due to ROS. It may be worth testing the sensitivity of the triple mutant to other sources of ROS, such as paraquat, as there is the potential that different results would be seen.

STM1808 has previously been shown to be important for virulence in the murine model, with the authors suggesting a role in protection against nitrosative stress in macrophages (Karlinsey et al. 2012). In this study, we showed the tellurite hypersensitive *ΔtehB ΔSTM1808 ΔyeaR* strain to be attenuated in IFN-γ activated macrophages. The fact that we see this attenuation only in IFN-γ activated macrophages and not in L-NAME treated macrophages, which will fail to produce the nitrosative burst, further implicates these proteins in NO defense. It would be of merit to repeat these experiments treating the macrophages both with IFN-γ and L-NAME

in order to confirm the differences we see between the IFN-γ and L-NAME samples are solely due to NO release and not other defensive mechanisms controlled by IFN-γ.

This work has raised a new area of research for the enigma of tellurite toxicity – the link between tellurite sensitivity and NO sensitivity; and here there are still many unanswered questions. Originally, was the primary role of these three Te^R proteins in protection against tellurite, but when over time the environmental conditions *Salmonella* encounters changed, these proteins were retained due to their secondary role in NO resistance? Or conversely are these proteins simply involved in NO detoxification and by chance have the ability to protect *Salmonella* against tellurite? If the latter is the case we perhaps would expect other tellurite resistance systems, (KlaABC, TerZABCDEF, Tmp, TrgAB and CysK*)* to contribute to NO detoxification and/or tellurite resistant bacteria such as *Corynebacterium diphtheriae*, *Streptococcus faecalis*, and some drug resistant *Staphylococcus aureus* strains (Summers and Silver 1978) to have increased resistance to NO.

There is one previous study linking nitrogen metabolism to tellurite, this links NarG, the nitrate reductase, to tellurite detoxification (Avazéri et al. 1997). They also identified an additional, unidentified, soluble protein which showed tellurite reductase activity. There was no tellurite sensitivity of the NarG mutant under anaerobic conditions, and the authors suggest this is due to the additional protein they identified; there is the potential that this is one of the proteins studied in this chapter. Avazéri *et al.,* did not indentify the mechanism of tellurite reduction by NarG and could not conclude whether this single protein is responsible for the total reduction of Te^4 to elemental tellurium, Te^0. The pathways of tellurite and nitrate reduction are somewhat similar and as such we propose the potential model shown in Figure 24.

This model suggests that *Salmonella* treats tellurite very similarly to nitrate, reducing it via the same pathway.

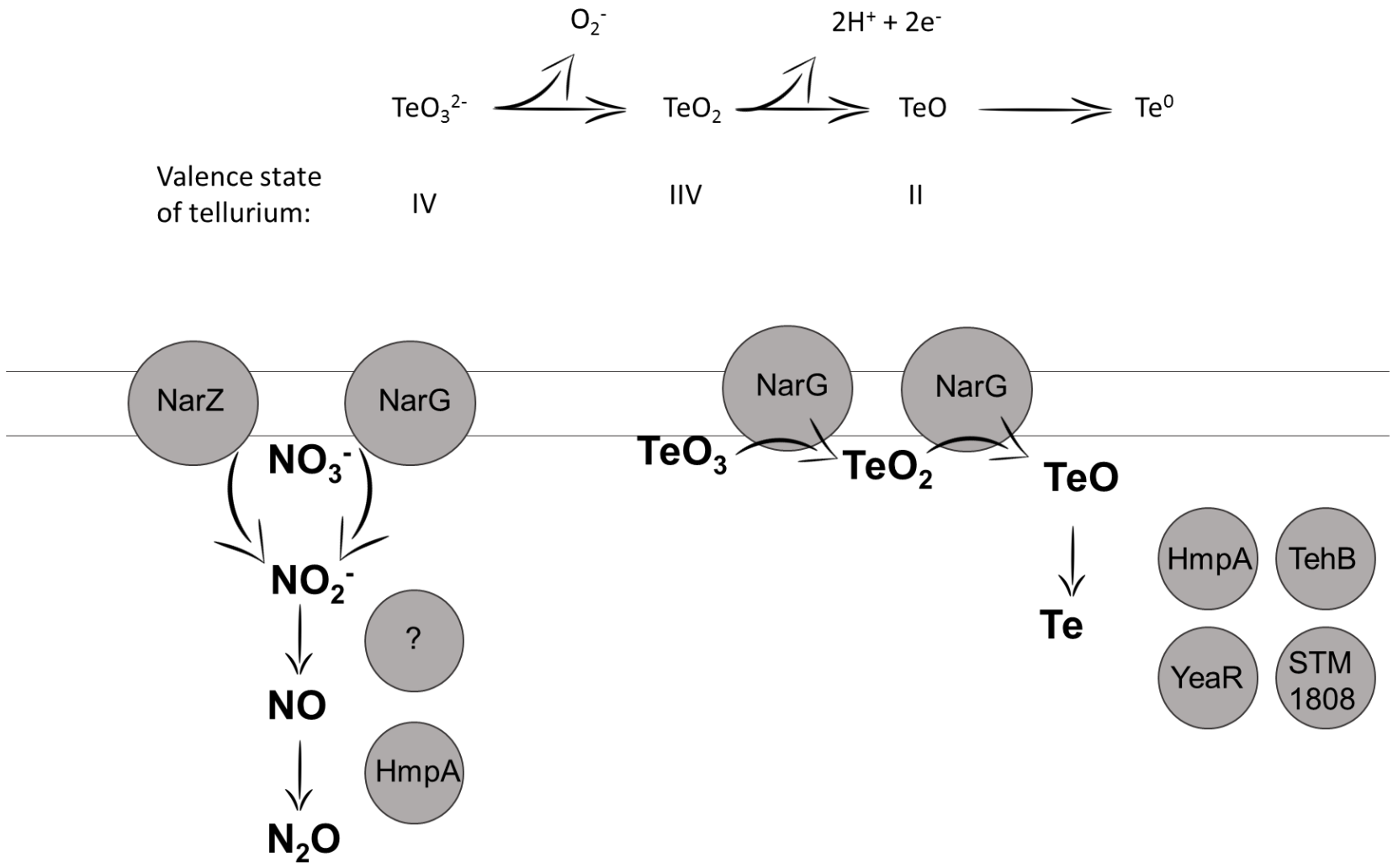


Figure 24 **Tellurite reduction model**

While this chapter has raised numerous further questions it has also achieved the original intention of this work, which was to characterize novel anaerobic nitric oxide detoxifiers. It is well known that the ability of *Salmonella* to survive within macrophages is vital to the progression of Typhoid fever (Page 25) and therefore that a strain which is unable to survive within this environment will not be able to progress into the systemic, fatal, stage of the disease. While STM1808 and TehB have been studied before in relation to nitric oxide detoxification (Karlinsey et al. 2012), their full effect, and therefore potential importance in this area, was not seen due to the functional overlap exhibited between these two proteins and TehB.

3.3.1 Future Work

The first thing that must now be done is protein biochemistry to confirm the role of YeaR, TehB and STM1808 in direct nitric oxide detoxification. This could be in the form of protein film voltammetry, a technique that has previously been used to quantify the NO reductase activity of NrfA from *E. coli* (van Wonderen et al. 2008). This technique works by immobilizing the enzyme onto an electrode, placing this in the potential substrate and monitoring any charge produced due to the flow of electrons (Angove et al. 2002).

Further work should be done to assess the potential for these three proteins in combination with others as a potential vaccine strain. The first steps would be to combine these three mutations with other proteins such as HmpA, NrfA and NorV and assess the strain's survival in macrophages. We would expect such a strain to have a very severe attenuation.

4. The role of CstA, Hcr and STM1273 in nitric oxide and tellurite resistance

4.1 Introduction:

Chapter 3 looked at the proteins transcribed by three genes which were upregulated in response to NO and importantly all had the same annotated function in tellurite resistance. This chapter looks at three further genes which were upregulated under the same conditions but at the time had not been confirmed to have a role in nitric oxide protection or detoxification. Background information on these three proteins, CstA, Hcr and STM1273 is given below.

Two microarray analyses by Arkenberg *et al.,* (personal communication) revealed upregulation of these three genes both when subjected to an exogenous bolus of nitric oxide (40 μM) or grown under denitrifying conditions which will cause *Salmonella* to release both NO and N_2O. *cstA* exhibited similar levels of upregulation in both of these analyses with a 4 fold increase from exogenous exposure and 7 fold during endogenous production. *hcr* was very highly upregulated under denitrifying conditions with a 77 fold increase in expression, but was also 7 fold upregulated by an exogenous NO bolus. STM1273 was upregulated 2.4 fold post exogenous NO exposure and not significantly changed when respiring on nitrate.

4.1.1 CstA

CstA has been described as a peptide transporter, contains eighteen transmembrane domains and is annotated as a carbon starvation protein, being cAMP-CRP (cyclic adenosine monophosphate-catabolite regulation protein) dependent in E. coli; that is when carbon levels drop cAMP levels rise and activate expression of the gene (Blum et al. 1990, Garai et al. 2015). Glucose starvation of E.coli results in a 6 fold increase in expression levels (Blum et al. 1990). CstA acts as a virulence factor in Salmonella; as a cstA mutant had a 67% reduction in C. elegans killing compared to WT. A cstA mutant was also 39% reduced in its ability to initiate polymorphonuclear leukocytes migration and 54% reduced in epithelial cell invasion (Tenor et al. 2004). There has been no study looking at this virulence further, for example in the mouse model. Most studies on cstA have looked at its regulation. CsrA, a global regulator, has been shown to translationally regulate CstA in E. coli, the main targets of CsrA regulation are proteins involved in central carbon metabolism, motility, and biofilm development (Dubey et al. 2003).

Another regulator is YjiY, in both *S*. Typhi and *S.* Typhimurium *cstA* has been shown to be the predominant gene regulated by this protein. In *E. coli* YjiY is regulated by the two-component system YehUT, which is conserved throughout the majority of enteric bacteria. Similarly, to YjiY transcriptome analysis identified *cstA* as the predominantly yehUT regulated gene in both *S*. Typhi and *S.* Typhimurium (Wong et al. 2013). The YehUT operon of *S*. Typhi has been found to contain a higher than average level of mutations, suggesting adaptive evolution is occurring; potentially indicating an importance for this operon (Wong et al. 2013).

4.1.2 Hcr

Hcr is transcribed in an operon with Hcp, a hybrid cluster protein. Hcr catalyses the reduction of the Hcp with NADH as the electron donor. In *E.* coli the two proteins are only produced when the bacteria is grown in the presence of nitrate and nitrite – suggesting a role in nitrogen metabolism (van den Berg, Hagen and van Dongen 2000, Overton et al. 2006). There has been suggestion that *hcr* functions as a hydoxylamine reductase and has peroxidase activity (Almeida et al. 2006, Wolfe et al. 2002).

A 2007 study tested the importance of Hcr for aerobic survival in LB in the presence of 3 mM GSNO (a NO donor) and saw no growth defect for a *S.* Typhimurium *hcr* mutant (Gilberthorpe et al. 2007). However, a role for Hcr in protection against NO-mediated respiration inhibition has been shown in *Salmonella*. Since HmpA is said to be the most important protein for NO detoxification, Karlinsey *et al.,* constructed their mutants in a Δ*hmpA* background to avoid this masking any functions, additionally a *nsrR* deletion was used to avoid any interactions of NsrR related genes. The growth profile of this Δ*nsrR* Δ*hmpA Δhcr* strain was monitored in LB containing 3mM Spermine-NONOate and a growth defect was seen (Karlinsey et al. 2012). In *E. coli*, Hcr has been confirmed to be a high affinity NO reductase during anaerobic growth (Wang et al. 2016).

NsrR has been confirmed to regulate *hcp-hcr* in both *E.* coli and *Salmonella* (Karlinsey et al. 2012, Filenko et al. 2007). In *E. coli*, *hcp-hcr, hmpA,* and *ytfE* are the three transcripts most tightly regulated by NsrR (Filenko et al. 2007).

The *hcp-hcr* operon is induced in activated RAW 264.7 macrophages but not by untreated macrophages or those where iNOS activity has been inhibited. (Kim, Monack and Falkow 2003). Infection studies using C57BL/6J mice surprisingly revealed increased killing of the mice for low doses of the *hcp* and *hcr* mutants (termed *nipA* and *nipB* respectively) (Kim et al. 2003). It was then noted by Karlinsey et al., that C57Bl/6J mice lack a functional Nramp1 locus and as such have reduced NO production, and so a study was conducted with C3H/HeN mice which do not lack this locus. This study inoculated mice with a 1:1 ratio of WT and the mutant and examined surviving bacteria in numerous organs. However, *hcp* mutants outcompeted the WT strain indicating the lack of *hcp* results in increased survival of *Salmonella* and corroborating the results of Kim *et al.* (Karlinsey et al. 2012).

4.1.3 STM1273

STM1273 lies 410bp downstream of *yeaR*, the role of which in nitric oxide and tellurite resistance was confirmed in Chapter 3. There is very little in the literature about this protein, however it was originally annotated as a putative nitric oxide reductase (McClelland et al. 2001). The RyhB sRNA have mainly been shown to affect translation of proteins requiring iron in response to low iron availability. One of the RhyB

homologs, *ryhB-2* overlaps with *STM1273*, leading to the suggestion that *STM1273* may be a target of RyhB regulation (Kim and Kwon 2013a, Kim and Kwon 2013b, Kim 2016). Previous work from the Rowley lab found no role for STM1273 anaerobically in the presence of 40 μM aqueous NO. NO sensitivity had not been looked at during aerobic growth. The fact that *STM1273* is only upregulated after a NO bolus and not during nitrate respiration suggests that it may be more important for exogenous rather than endogenous NO detoxification.

4.2 Aims

Based on the results of the previous chapter, which found a link between NO and tellurite protection both aerobically and anaerobically in *Salmonella*, this chapter will further assess the importance of three proteins in both NO and tellurite resistance.

- Determine the role of CstA, Hcr and STM1273 in NO protection, aerobically and anaerobically.
- Assess the importance of CstA, Hcr and STM1273 in macrophage survival
- Challenge *ΔcstA*, *Δhcr* and *ΔSTM1273* with potassium tellurite to assess if they play a role in protection

4.3 Results

4.3.1 *Δhcr ΔSTM1273* and *ΔcstA* grow comparably to WT in LB both aerobically and anaerobically

Deletion mutants (Arkenberg, personal communication) were first tested for any general growth defects on LB both aerobically and anaerobically. All three mutant strains grew comparably to WT (Figure 25).

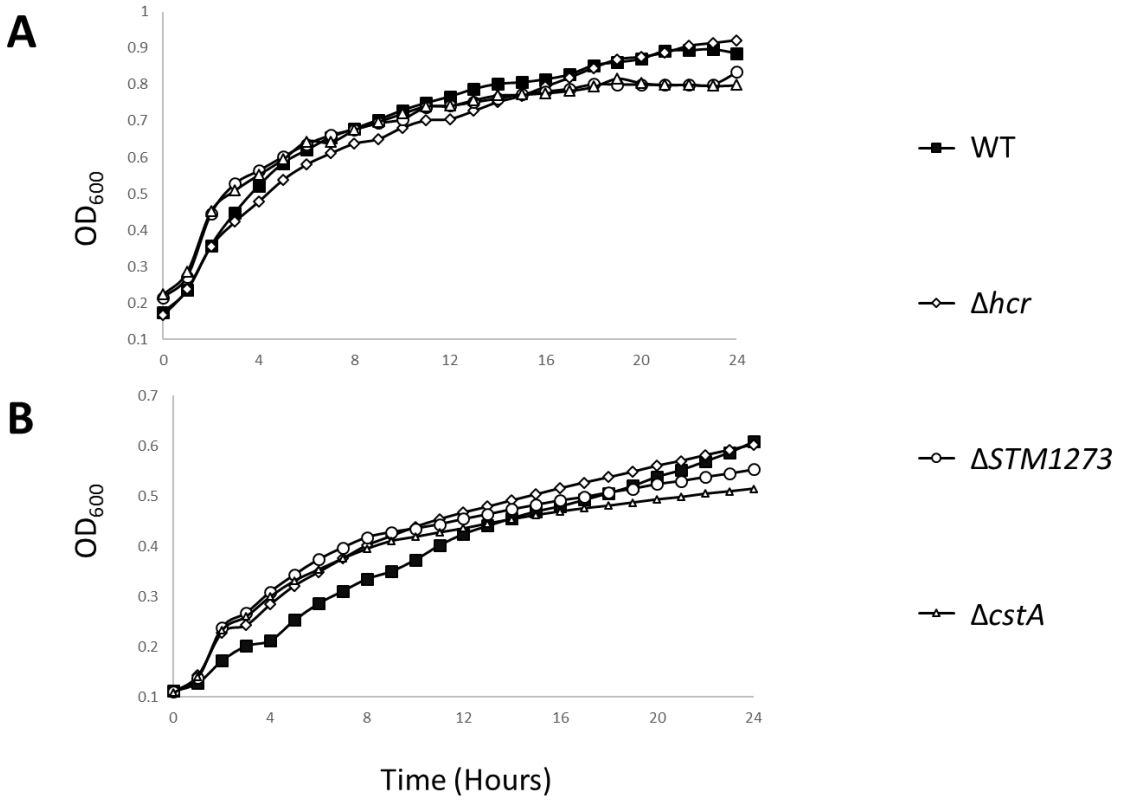


Figure 25 **The three mutant strains, Δ*hcr*, Δ*STM1273* and Δ*cstA* grow comparably to WT in LB.** Conducted both aerobically (A) and anaerobically (B). Strains were grown for 24 hours in 1 ml LB in a SpectraMax plate reader. Data shows 3 repeats.

4.3.2 STM1273, CstA and Hcr are important for nitric oxide protection both aerobically and anaerobically

In order to determine the contribution of STM1273, CstA and HmpA to the survival of *Salmonella* when challenged with NO, deletion mutants of *STM1273*, *cstA* and *hcr* were grown in the presence of 5 mM Deta NONOate both aerobically and anaerobically (Figure 26). Aerobically the three strains, *ΔSTM1273*, *ΔcstA* and *Δhcr* showed similar levels of growth, with the final ODs being 67%, 61% and 66% of WT's, respectively. Anaerobically, again, the three strains *ΔSTM1273*, *ΔcstA* and *Δhcr* showed similar levels of growth to each other, with the final ODs being 72%, 71% and 69% of WT's. This therefore confirms the role of all three proteins in both aerobic and anaerobic exogenous nitric oxide protection.

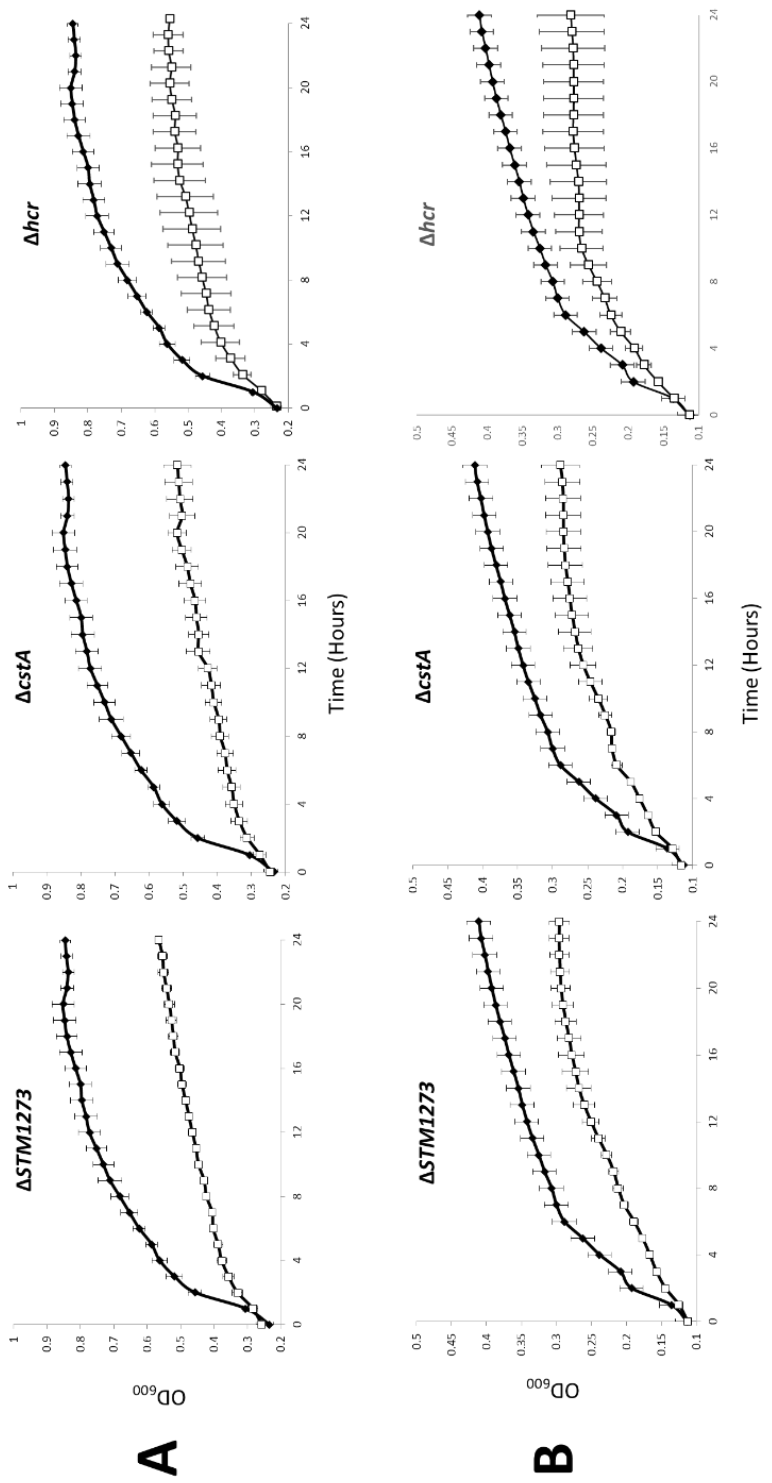


Figure 26 **STM1273, CstA and Hcr are important for nitric oxide protection both aerobically and anaerobically.** Strains were grown both aerobically (A) and anaerobically (B) in 1ml LB in a SpectraMax plate reader for 24 hours, with the addition of 5 mM Deta NONOate. Solid black circles indicate the WT and white circles the indicated mutant strain. All strains have a minimum of 3 repeats and SE is shown.

4.3.3 Nitric oxide accumulates in *Δhcr* in comparison to WT when grown under denitrifying conditions

When grown in minimal media with the addition of nitrate and glycerol, *Salmonella* will use the provided nitrate as a terminal electron acceptor and undergo denitrification. Nitric oxide is produced as an intermediate of denitrification. We would therefore expect a strain lacking a gene which is important in the reduction of nitric oxide to accumulate greater levels than WT. When cultured anaerobically on M9 with 20 mM nitrate, both *ΔcstA* and *ΔSTM1273* produced slightly lower levels of NO than WT at 40 hours, suggesting they play no direct role in NO detoxification (Figure 27). There was however a near 2-fold significant increase in the level of NO which accumulated in the *Δhcr* mutant suggesting that this protein plays a direct role in the reduction of NO (Figure 27).

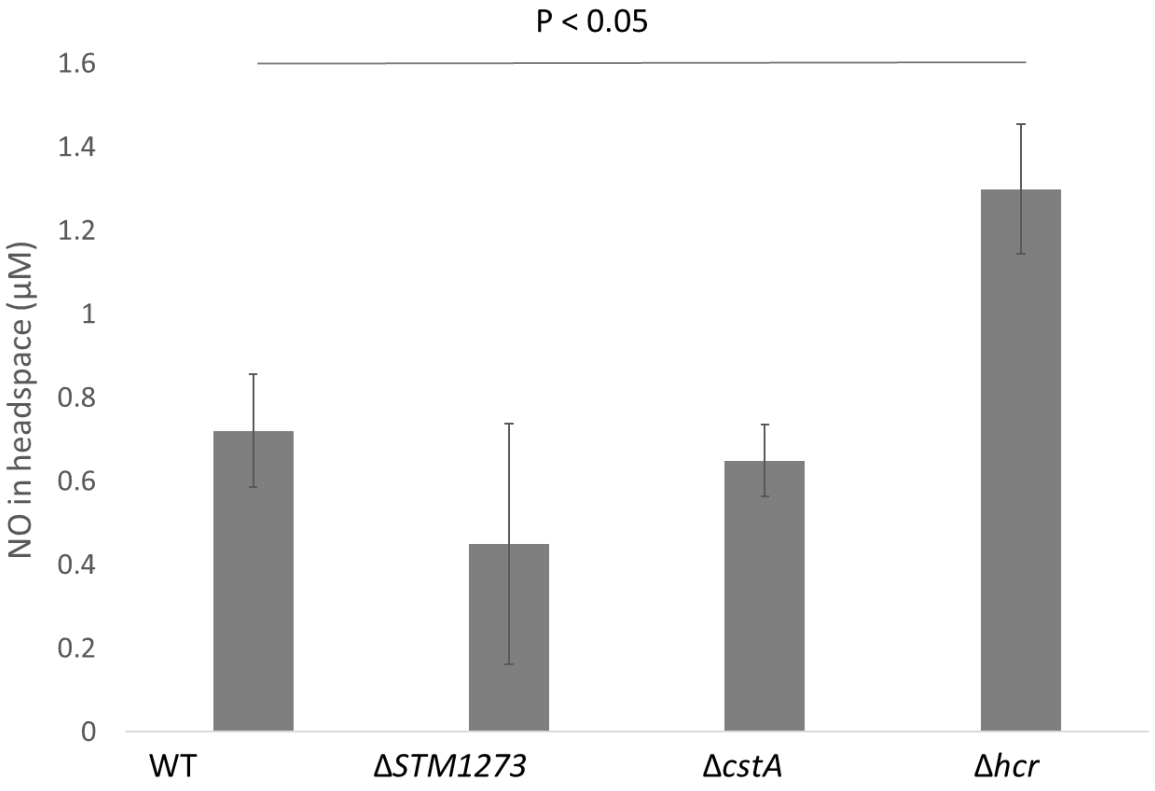


Figure 27 **Nitric oxide accumulates in Δ*hcr* in comparison to WT when grown under denitrifying conditions.** Strains were grown in M9 minimal media with the addition of 20mM NaO_3 and 10mM glycerol. Nitric oxide from the headspace was measured at 40 hours. All strains have a minimum of 3 repeats, SE is shown and a student's T Test was used.

4.3.4 *Δhcr* and *ΔcstA* are significantly attenuated in activated macrophages

Macrophages release NO as an integral part of the host response via iNOS, the inducible nitric oxide synthase. iNOS can be upregulated by interferon gamma (IFN-γ) or inhibited by addition of NG-nitro-L-arginine methyl ester (L-NAME) (Pfeiffer et al. 1996). Therefore, IFN-γ-activated macrophages release higher, more physiologically relevant, levels of NO and L-NAME-treated macrophages will release low or no NO. Bacterial counts were taken 2 and 24 hours post macrophage infection and the fold reduction in numbers is shown (Figure 28). As would be expected, all strains had increased survival when the macrophages were treated with L-NAME in comparison to IFN-γ. There is a significantly greater reduction in bacterial numbers for *ΔcstA* in comparison to WT, this would suggest that this protein plays a role in survival inside macrophages which is not based on NO detoxification. All three strains, *Δhcr, ΔcstA* and *ΔSTM1273* show a virulence defect in IFN-γ treated macrophages. The WT strain has a 13.8 fold reduction in cfu/ml of *Salmonella* between 2 and 24 hours, this increases to 22.2 fold for *ΔcstA, 22.3* fold for *ΔSTM1273* and 26.8 fold for *Δhcr.*

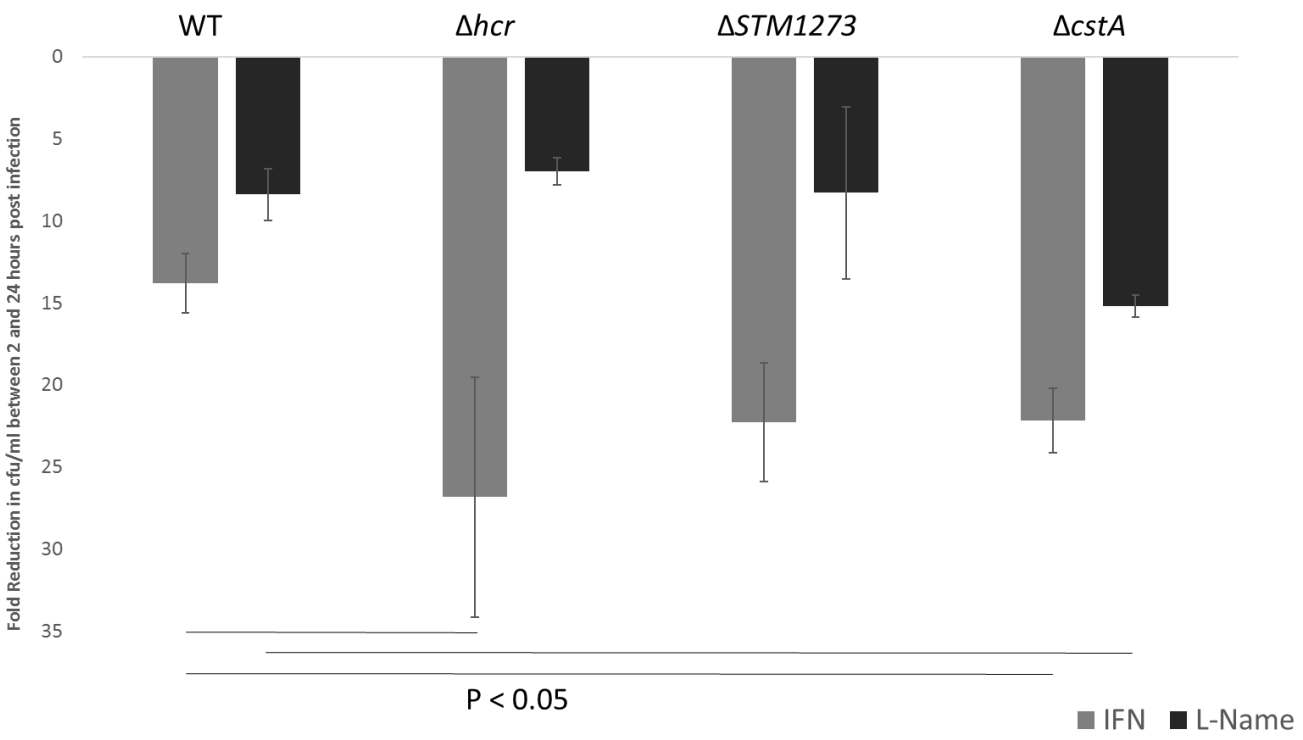


Figure 28 **Hcr and CstA are important for survival within activated macrophages. Fold** change in cfu/ml between 2 and 24 hours post infection is shown for L-NAME treated (filled) and IFN-y activated (shaded) RAW 264.7 macrophages. 4 repeats were used and SE is shown. A student's t-test was used.

4.3.5 STM1273, Hcr and CstA all offer *Salmonella* protection against potassium tellurite in the presence and absence of oxygen.

Following on from Chapter 3, once Hcr, CstA and STM1273 had been shown to be important for nitric oxide protection we wanted to see if like other known nitric oxide detoxification systems, YtfE, NorV, NrfA, HmpA, there was a role for these proteins in potassium tellurite protection. All three mutants exhibited a slight growth defect both aerobically and anaerobically in the presence of potassium tellurite (Figure 29). It should be noted in contrast that YtfE, NorV and NrfA only appear to play a role in Te^R anaerobically, and HmpA only aerobically (Figure 22).

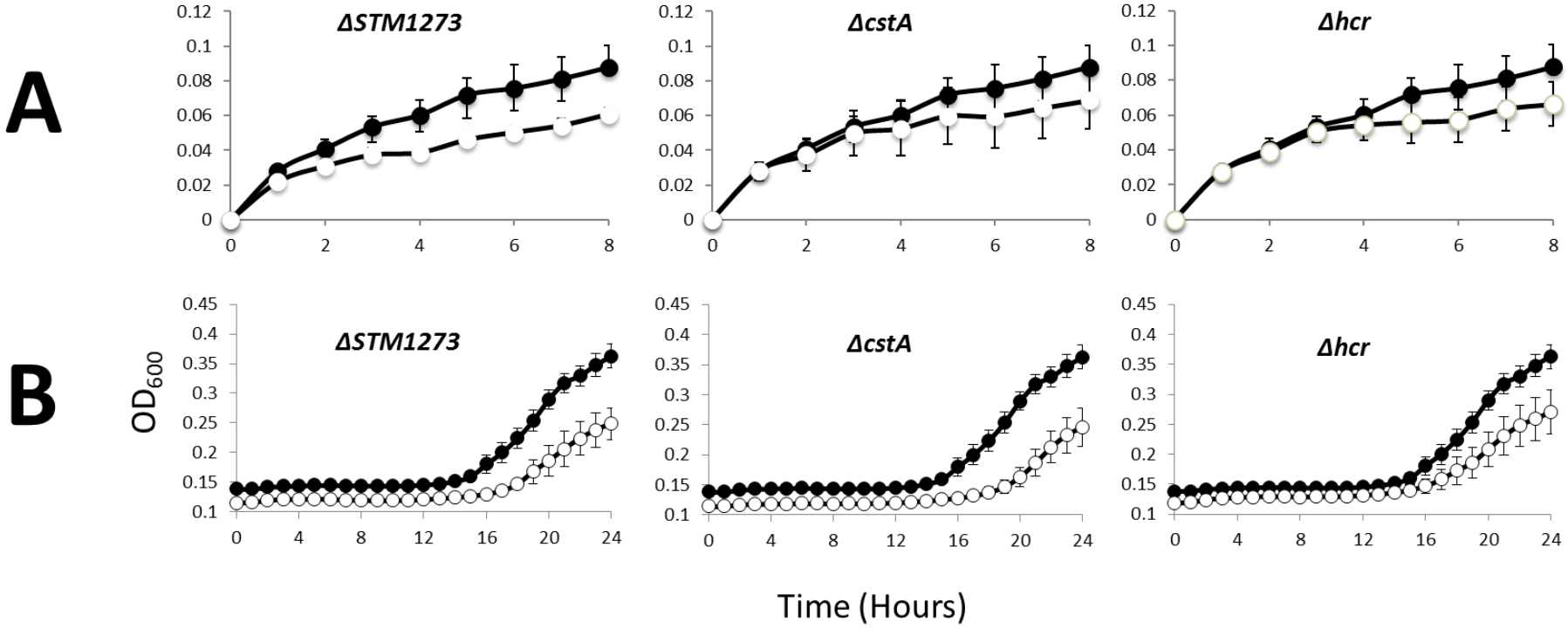


Figure 29 **STM1273, CstA and Hcr all play a role in tellurite protection, both aerobically and anaerobically.** Strains were grown aerobically in 50ml LB (A) or anaerobically in 1ml LB (B) with the addition of 5 μM potassium tellurite.

4.4 Discussion

The importance for *Salmonella* to efficiently detoxify nitric oxide and the systems in place to do this has been discussed in detail in the introduction (Page 33) and expanded upon in Chapter 3.

Of the three proteins studied in this chapter, Hcr has been the most well characterised, especially in regards to NO protection. It is known to be NsrR regulated in *Salmonella* which infers a strong likelihood to being involved in NO protection. Additionally, Hcr has been shown to play a role in NO-mediated respiration inhibition (Karlinsey et al. 2012) but not in direct NO protection aerobically (Gilberthorpe et al. 2007). In *E. coli,* Hcr has been shown to encode a NADH oxidoreductase, and be transcribed in an operon with Hcp, the hybrid cluster protein; additionally it was seen that both proteins are needed in combination order to reduce NO in anaerobic conditions (Wang et al. 2016, van den Berg et al. 2000, Filenko et al. 2007).

In this chapter, we have shown a role for Hcr in both aerobic and anaerobic growth – with growth defects compared to WT in both conditions in the presence of 5mM deta NONOate. Additionally, an experiment looking at the levels of NO in the headspace of an *hcr* mutant growing under denitrifying conditions showed a near 2-fold increase in comparison to WT. This indicates a direct role of Hcr in nitric oxide detoxification. Finally, *Δhcr* was significantly attenuated in activated macrophages, with a 26.8 fold reduction in bacterial numbers during infection compared to 13.8 fold for the WT strain. It has been previously shown that *hcr* is upregulated in RAW 264.7 macrophages and hence it would be expected the protein would play a role in survival (Kim et al. 2003).

As noted in the introduction of this chapter very little is known about the STM1273 protein, but one thing of note is that is it situated just downstream of YeaR, separated only by one gene, YoaG. As shown in Chapter 3, YeaR is important both for nitric oxide and tellurite protection, and while being closely situated on the genome is not evidence alone that two proteins would have similar function, it is often common. It is clear from Figure 26 that STM1273 plays a role in protection against NO both aerobically and anaerobically in *Salmonella.* In contrast to Hcr however this did not correlate to an increase in the NO that accumulated under denitrifying conditions (Figure 27), which would indicate that the role STM1273 plays in protection against

NO is not in directly detoxifying it. It could however be the case that the loss of STM1273 results in the upregulation of one or more other NO detoxification systems and hence causes a decrease in NO emissions; therefore, this result should not be used to totally disregard the putative function of STM1273 as a nitric oxide reductase. While the *STM1273* mutant did not fare significantly worse than WT in activated macrophages, it did show an increase in bacterial death. It is therefore possible that STM1273 plays a minor role in the survival of *Salmonella* inside macrophages, which could be further highlighted in combination mutant strains.

CstA, annotated as a carbon starvation protein, also appears to play a role in NO protection, both aerobically and anaerobically (Figure 26). The *ΔcstA* strain accumulated similar levels of NO in comparison to WT when grown in minimal media supplemented with nitrate, as with STM1273, this does not necessarily mean that CstA does not directly detoxify NO, however it may also be the case that the protein is instead involved in protecting *Salmonella* from some of the toxic effects of NO.

The results from the macrophage infection experiment have two implications for CstA. Firstly, the strain has significantly reduced survival in activated macrophages in comparison to the WT strain, suggesting that the protein is important for the survival in normal, activated macrophages. Secondly, in L-NAME treated macrophages, where iNOS is inhibited and hence NO is not produced, the *cstA* mutant also had significantly reduced survival. This suggests that CstA is important for survival in macrophages not solely because of its ability to provide protection against NO.

The most likely reason for this is the general stress response function of CstA. The stress response sigma factor, σ^S, is thought to upregulate CstA (Dubey et al. 2003). There are five alternative sigma factors in *Salmonella*, σ^S is primarily stimulated by nutrient starvation or by the bacteria entering stationary phase (Hengge-Aronis 2002). As a general stress response regulator σ^S is known to be important for numerous stresses, including a number which would be present while *Salmonella* resides within macrophages and indeed has been shown to be important for survival within the environment of the macrophage. Specifically, σ^S is important for starvation, including nitrogen and carbon limitation (McCann, Kidwell and Matin 1991, Mulvey et al. 1990, Spector and Cubitt 1992, Spector 1998), oxidative stress (Lange and Hengge-Aronis 1991) and osmotic shock (McMeechan et al. 2007). Carbon starvation proteins have been shown to be induced during macrophage growth (Abshire and Neidhardt 1993)

and it is known that Glucose and Glycolysis are necessary for a successful macrophage (and mouse) infection by *Salmonella* (Bowden et al. 2009, Bowden et al. 2010). In addition, σ^S, has specifically been shown to be important for the survival of *Salmonella* within the macrophage (Fang et al. 1992).

Lastly, we tested the three mutants, *Δhcr ΔcstA* and *ΔSTM1273,* for their survival in the presence of potassium tellurite both aerobically and anaerobically. All three strains showed a mild growth defect in comparison to WT under both conditions. This strengthens the argument made in Chapter 3 that there is a correlation between tellurite resistance and NO resistance in *Salmonella*; however, it does not bring any new information as to why or how this is the case.

In summary, this chapter has confirmed the role of the three proteins, CstA, Hcr and STM1273 in nitric oxide protection of *Salmonella* both aerobically and anerobically. The role of Hcr seems the most clear, with a distinct increase in the amount of NO which accumulates, and reduced survival in activated macrophages. While the role that STM1273 and CstA play is slightly less concrete, there is good evidence that the proteins are involved in the NO response and additionally with macrophage survival. As with the proteins in Chapter 3, protein biochemistry, including protein film voltammetry could be used to ascertain whether these proteins directly reduce NO.

5 Contribution of Vitamin B_{12} dependent and independent methionine synthases to anaerobic nitrate respiration

5.1 Introduction

This chapter examines a potential link between the production of two compounds produced by *Salmonella*, vitamin B_{12} and nitrous oxide – both of which should seemingly come at a cost to the bacteria.

The overarching aim of this chapter will be to determine if nitrous oxide production by *Salmonella* contributes to pathogenesis and is based on two major observations. The first is a result of a combination of studies by Rowley et al, 2012, Streminska et al, 2012 and Runkel et al (in prep) who identified that nitrous oxide production by *Salmonella* Typhimurium cultured in nitrate sufficient/glycerol limited chemostats is an order of magnitude higher than that produced by related non-pathogenic *E. coli* strains (Figure 3). The second being the observation by Sullivan et al, that N_2O accumulation in *Paracoccus denitrificans nosZ* mutants (N_2O genic) induces a switch from vitamin B_{12}-dependent to vitamin B_{12}-independent biosynthetic pathways (Sullivan et al. 2013). Nitrate respiration and therefore possibly N_2O emissions, are particularly relevant to two infection scenarios; the first in the inflamed intestine where nitrate is produced as part of the host inflammatory response (Winter et al, 2010), and, the second in the intracellular environment of the macrophage.

As described in more detail in the introduction (1.8 Denitrification in *Salmonella*, Page 30), as a facultative anaerobe *Salmonella* has the ability to respire on nitrate, and in doing so releases nitrous oxide as an end product. When grown under denitrifying conditions in a chemostat *Salmonella* has been shown to produce N_2O at measurable amounts of ≈1600 µM, in comparison to *E. coli* which releases > 100 µM (Figure 3). This is a surprising phenotype as due to its interaction with vitamin B_{12}, nitrous oxide is deemed toxic.

The synthesis and requirements of vitamin B_{12} in *Salmonella*, primarily in methionine synthesis, are described in the introduction (Page 39). This chapter will look at the interactions between vitamin B_{12} and nitrous oxide and additionally the regulation of vitamin B_{12} and methionine synthesis.

The 17 kb *cob* operon from which vitamin B_{12} is synthesised is grouped into three clusters: *cobI*, *cobIII*, *cobII* (Rondon and Escalante-Semerena 1996, Chen et al. 1995b). The *cob* operon is located adjacent to the *pdu* operon (Figure 30) which codes for propanediol dehydratase both of which are induced by propanediol. Furthermore propanediol induces transcription of *pocR,* a gene encoding the positive regulator of the *cob* operon, PocR (Rondon and Escalante-Semerena 1996). The expression of *pocR* is under the control of two regulators, Crp and ArcA, both classed as global regulators of genes in *Salmonella* (Ailion, Bobik and Roth 1993).

Crp is the receptor protein for cyclic AMP (cAMP) and is the global regulator for genes involved in carbon source utilisation in the absence of glucose. With over 200 targets in *E. coli* Crp is thought to regulate a wide range of proteins involved in transport of carbon sources as well as their utilisation (Shimada et al. 2011). Since it is known that PocR is important for the regulation of the *pdu* operon required for the use of the carbon source propandediol, it is logical that this operon is under the control of this regulator. ArcA, Aerobic Respiratory Control, is important for sensing oxygen levels within the cell. The regulon of ArcA in *E. coli* is well characterised but fewer studies have been conducted in *Salmonella* (Salmon et al. 2005, Liu and De Wulf 2004). In *Salmonella* ArcA either directly or indirectly targets nearly 400 genes, including those required for propanediol utilisation and ethanolamine utilisation; which is not the case in *E. coli* (Evans et al. 2011).

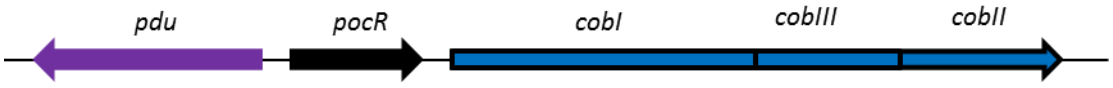


Figure 30 **The organisation of the *cob* operon of vitamin B_{12} synthesis with respect to the *pocR* gene and *pdu* operon.** The *cob (*vitamin B_{12} synthesis) and *pdu* (propanediol dehydratase) operons are both induced by PocR, the product of the *pocR* gene. (Diagram not to scale).

Despite studies demonstrating that deletion of *pocR* results in a downregulation of the *cob* and *pdu* operons (Bobik, Ailion and Roth 1992), the precise regulatory mechanisms of the *cob* operon and the importance of PocR remain unclear.

It is known that the presence of oxygen (as little as 2kPa) is enough to inhibit the *cob* genes but the exact mechanism is unclear. Additionally, it has been shown that anaerobic respiration as opposed to anaerobic glucose fermentation results in higher transcription of the operon; this is partially due to cAMP levels. The production of B_{12} however is not dependent on cAMP and the compound is possibly more important in establishing anaerobic conditions than in direct regulation (Escalante-Semerena and Roth 1987).

5.2 Aims:

This chapter aims to assess the impact of both exogenous and endogenous N_2O on *Salmonella* growth, with specific attention to the activity of methionine synthesis. In doing so we aim to provide an explanation for the synthesis of both N_2O and vitamin B_{12} in *Salmonella.*

Specifically, this will be achieved by:

- Creation of mutants in MetE, MetH, MetR.
- Monitoring survival of mutants under denitrifying conditions and in the presence of exogenous N_2O.
- Determining the importance of PocR for vitamin B_{12} synthesis.
- Investigating the importance of these proteins for *Salmonella* survival in macrophages.

5.3 Results

5.3.1 Construction of *metE* and *metR* truncated mutants.

Figure 31 **Position of *metE* and *metR* on the *Salmonella* SL1344 genome**

The vitamin B_{12} independent methionine synthase, MetE, and MetR, the activator for both *metE* and *metH,* lie next to each other on the genome, with 1202bp separating them (Figure 31). They are transcribed in opposite directions and therefore it is possible that their promoter sites are contained within each other. If this were the case it would mean that a complete deletion of *metE* might effectively result in a *metE metR* double mutant, and therefore MetH (reliant on the activator MetR unless exogenous B_{12} is present) would also not be functioning. Although promoter mapping in *E. coli* suggests this is not likely to be the case (Figure 32), to be sure that both the *metE* and *metR* mutants are true single mutants, truncated mutants of both genes have been produced where approximately 500bp at the 5' prime region of each gene have been left unaltered (Figure 33,

Figure 34).

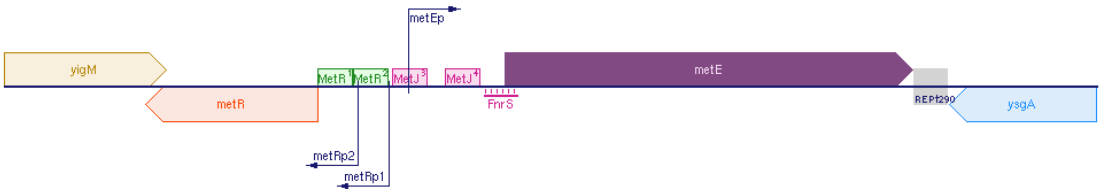


Figure 32 **MetE and MetR promoter site mapping in *E. coli*** (taken from biocyc.org)

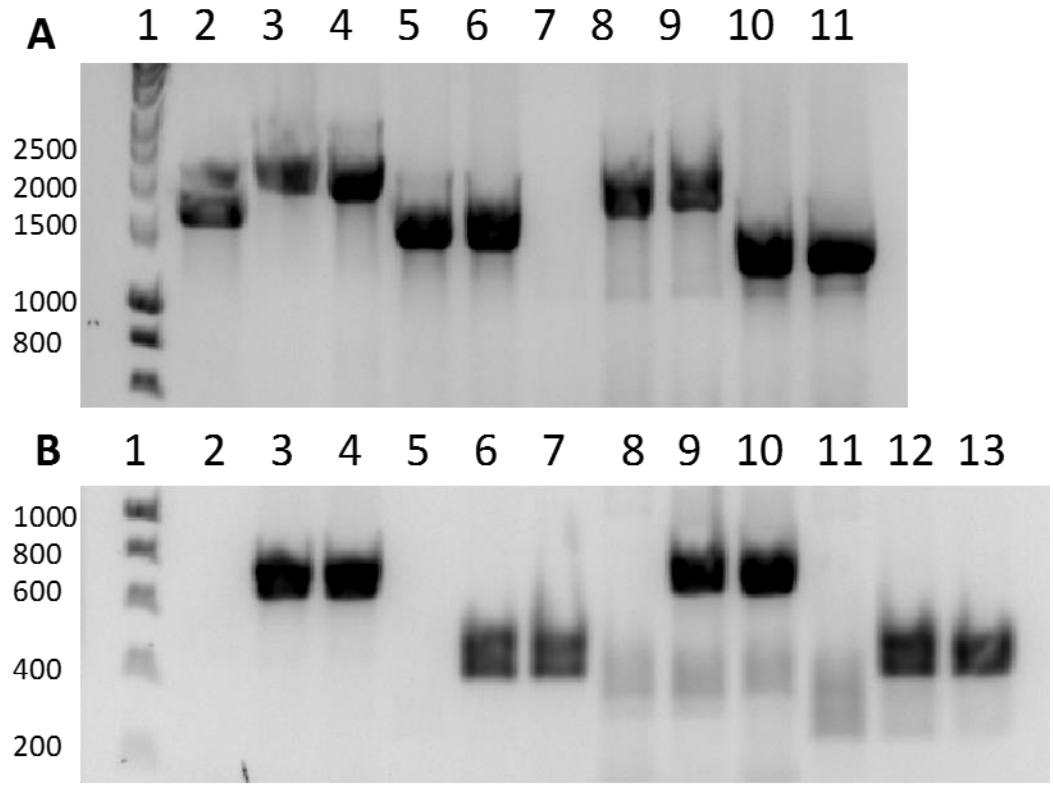


Figure 33 **PCR Verification of truncated *metE* and *metR* mutants.** A: Lane 1: Hyperladder. Lanes 2-6: External *metE* primers. Lane 2: WT, expected size 1500; Lane 3: *metE* Kan 2, expected size 2130; Lane 4: *metE* Kan 6, expected size 2130; Lane 5: *metE* Cm 2, expected size 1730; Lane 6: *metE* Cm 8, expected size 1730. Lanes 7-11: External *metR* primers. Lane 7: WT, expected size 795; Lane 8: *metR* Kan 1, expected size 2040; Lane 9: *metR* Kan 5, expected size 2040; Lane 10: *metR* Cm 2, expected size 1640; Lane 11: *metR* Cm 4, expected size 1640. B: Lane 1: Hyperladder. Lanes 2-4: External *metE* forward primer, internal pkD4 reverse primer. Lane 2: WT, expected no band; Lane 3: *metE* Kan 2, expected size 748; Lane 4: *metE* Kan 6, expected size 748. Lanes 5-7 External *metE f*orward primer, internal pkD3 reverse primer. Lane 5: WT, expected no band. Lane 6: *metE* Cm 2, expected size 464; Lane 7: *metE* Cm 8, expected size 464. Lanes 8-10: External *metR* forward primer, internal pkD4 reverse primer. Lane 8: WT, expected size, no band; Lane 9: *metR* Kan 1, expected size 748; Lane 10: *metR* Kan 5, expected size 748; Lanes 11-13: External *metR* forward primer, internal pkD3 reverse primer. Lane 11: WT, expected size, no band; Lane 12: *metR* Cm 2, expected size 464; Lane 13: *metR* Cm 4, expected size 464.

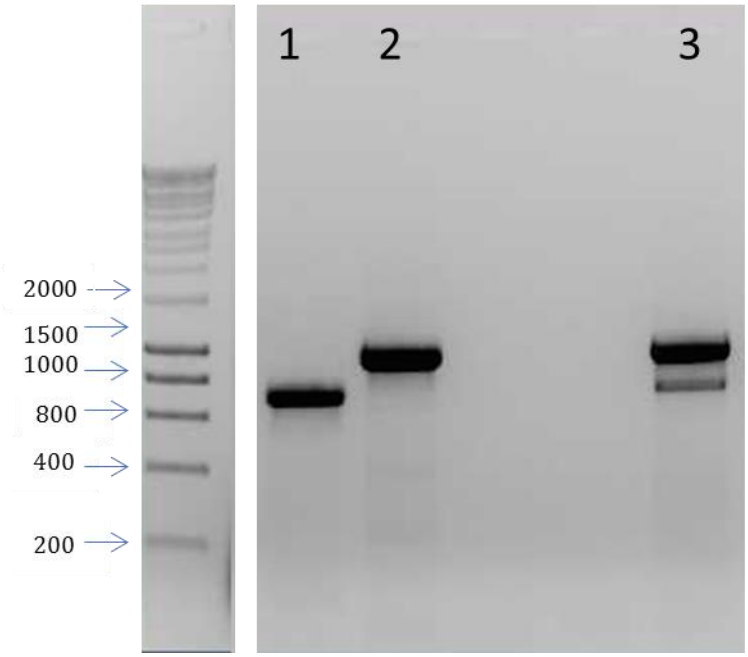


Figure 34 **Confirmation of mutant construction.** PCR confirmation of single mutants as follows: Lane 1:*MetH* (Cm), *metH* External, pkD3 Internal, 753; Lane 2: *MetH*(Kan), *metH* External, pkD4 Internal,1037bp; Lane 3: *WT, metH* External, *metH* Internal, 1070bp

5.3.2 All mutants grow like WT in LB aerobically

The methionine related mutants were grown alongside WT SL1344, aerobically, in rich LB media, to confirm the absence of any general growth defect (Figure 35). The concentration of methionine in LB broth has been shown to be between 2 and 6 mM (0.3-0.9 mg/ml) (Sezonov, Joseleau-Petit and D'Ari 2007), at least two fold higher than the amount required for supplementation of a known methionine autotroph, 150 µg/ml (Urbanowski et al. 1987).

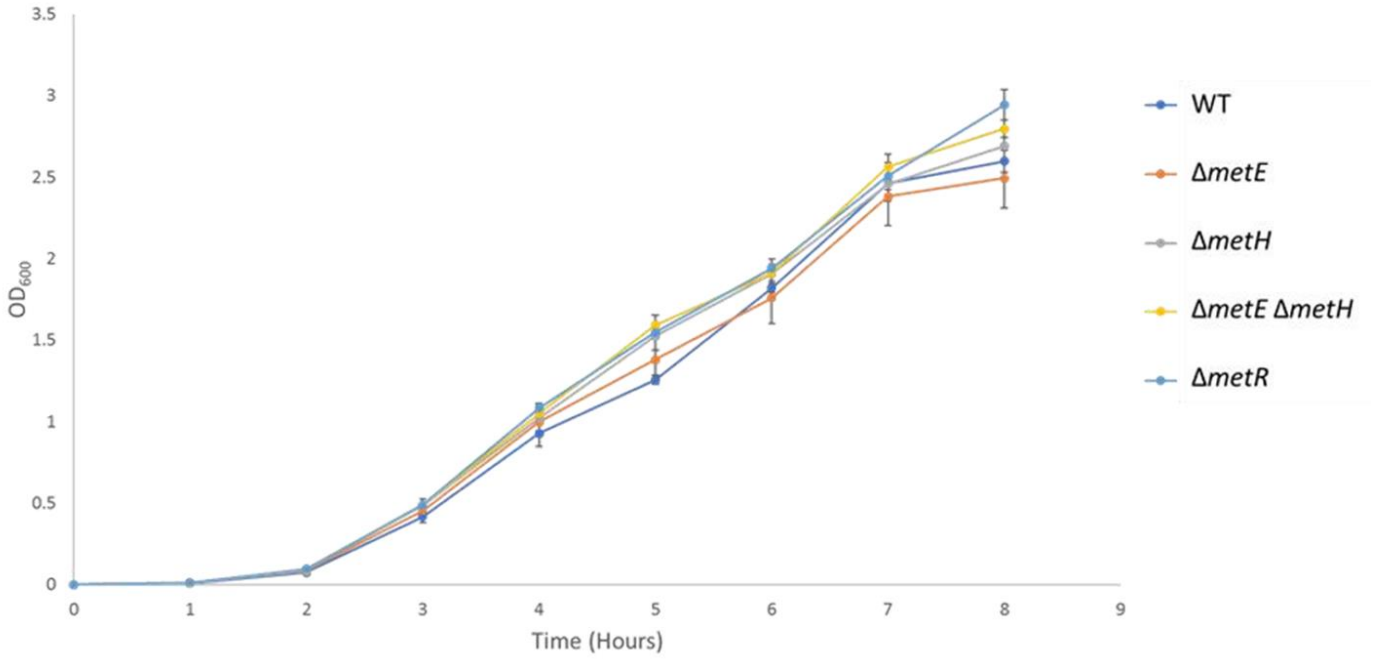


Figure 35 **All mutant strains grow at similar rates to WT aerobically in LB broth.** Strains were cultured in 50ml LB. Results are shown as means ± standard error of the mean from two separate experiments performed in duplicate.

5.3.3 *ΔmetE* and *ΔmetR* are attenuated in aerobic minimal media without the addition of vitamin B_{12} or methionine

Salmonella is only able to synthesis vitamin B_{12} anaerobically, therefore in aerobic conditions where exogenous methionine is lacking, the vitamin B_{12} independent methionine synthase, MetE, is required for survival (Figure 36). Three of the mutants, *ΔmetE*, *ΔmetE ΔmetH,* and *ΔmetR* are therefore completely attenuated when grown in M9 minimal media aerobically (Figure 36). When supplemented with either vitamin B_{12} (100 µg/ml) or methionine (30 µg/ml) this growth defect is fully restored to WT levels. The addition of methionine results in a growth benefit to all strains (in comparison to the addition of B_{12} and for WT and *ΔmetH* which do not possess growth defects under any conditions). This suggests there is a noticeable metabolic expense of the synthesis of methionine by MetE.

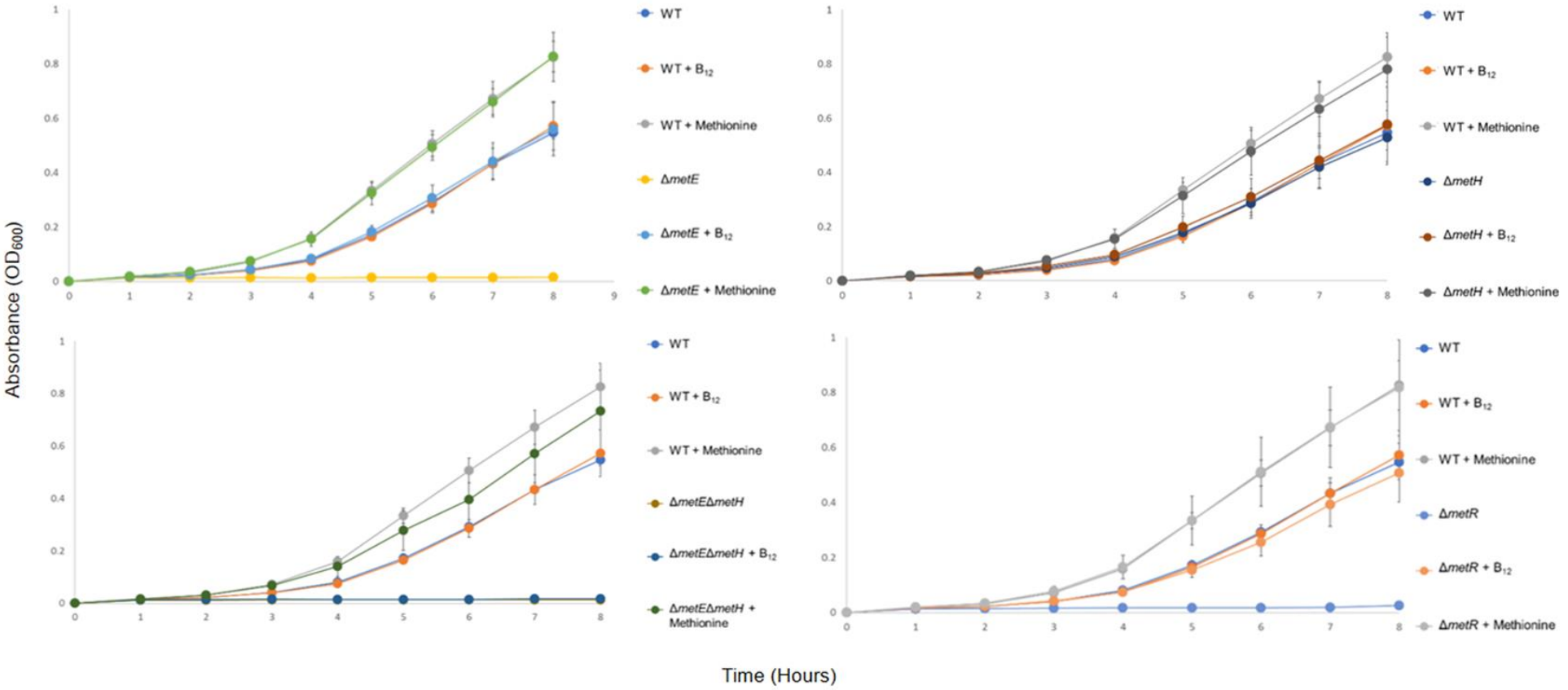


Figure 36 ***ΔmetE* and *ΔmetR* are attenuated in aerobic minimal media without the addition of vitamin B_{12} or methionine.** Strains were grown aerobically in 50ml M9 media + glucose with the addition of 100 µg/ml vitamin B_{12} and 30 µg/ml methionine where indicated. All strains have 3 repeats, and standard error is shown.

5.3.4 Vitamin B_{12} is toxic to *Salmonella*, but not due to cobalt toxicity

In order to chemically complement the B_{12} auxotrophic strain *ΔmetE*, an appropriate concentration of vitamin B_{12} needed to be determined. Vitamin B_{12} was initially added to anaerobic M9 cultures at 1 mg/ml as per (Sullivan et al. 2013) and while this resulted in restoration of growth of *ΔmetE* it inhibited growth of the SL1344 WT strain (data not shown). A range of concentrations of vitamin B_{12} were tested in order to determine a concentration where the WT strain and *ΔmetE* mutant grew comparably, this was found to be 0.05 mg/ml, 20 fold less than was used for the *Paracoccus* experiments (Figure 37).

Since Vitamin B_{12} has a cobalt ion at its centre we thought the Vitamin B_{12} toxicity being seen may be due to a cobalt toxicity. A previous study has put the MIC of cobalt at 18 µg/ml for *S. enterica* LT2 (Thorgersen and Downs 2009). Since cobalt makes up 4.3% of vitamin B_{12} when B_{12} is added at 1 mg/ml this equates to 0.043 mg/ml cobalt – however this is of course protected within the molecule. *Salmonella* was grown in the presence of a range of concentrations of cobalt from 0.043 mg/ml up to 1 mg/ml (far in exceeding that added with 1 mg/ml vitamin B_{12}) with no defect seen (Figure 38). We therefore conclude that the toxicity seen when *Salmonella* SL1344 WT is grown in the presence of vitamin B_{12} is not due to cobalt toxicity.

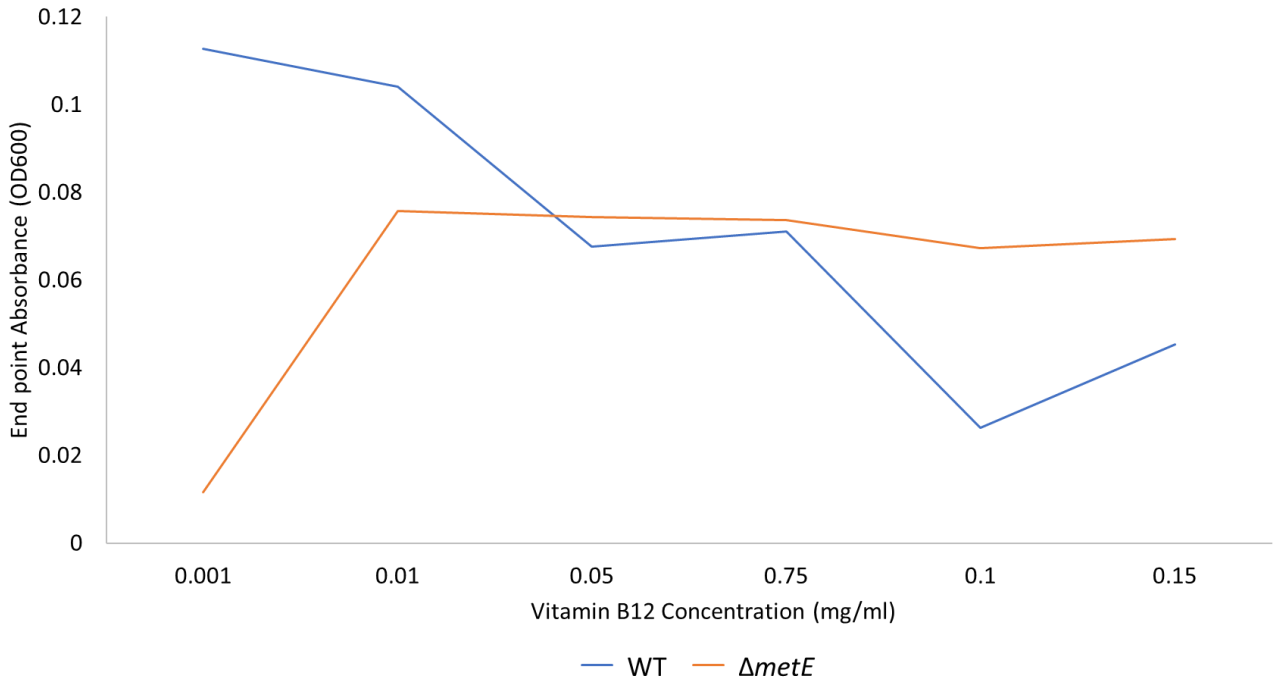


Figure 37 **Vitamin B_{12} exhibits a toxic effect on WT *Salmonella*.** Strains were grown in nitrate-sufficient, glycerol-limited M9 minimal media supplemented with vitamin B_{12} as indicated for 48 hours.

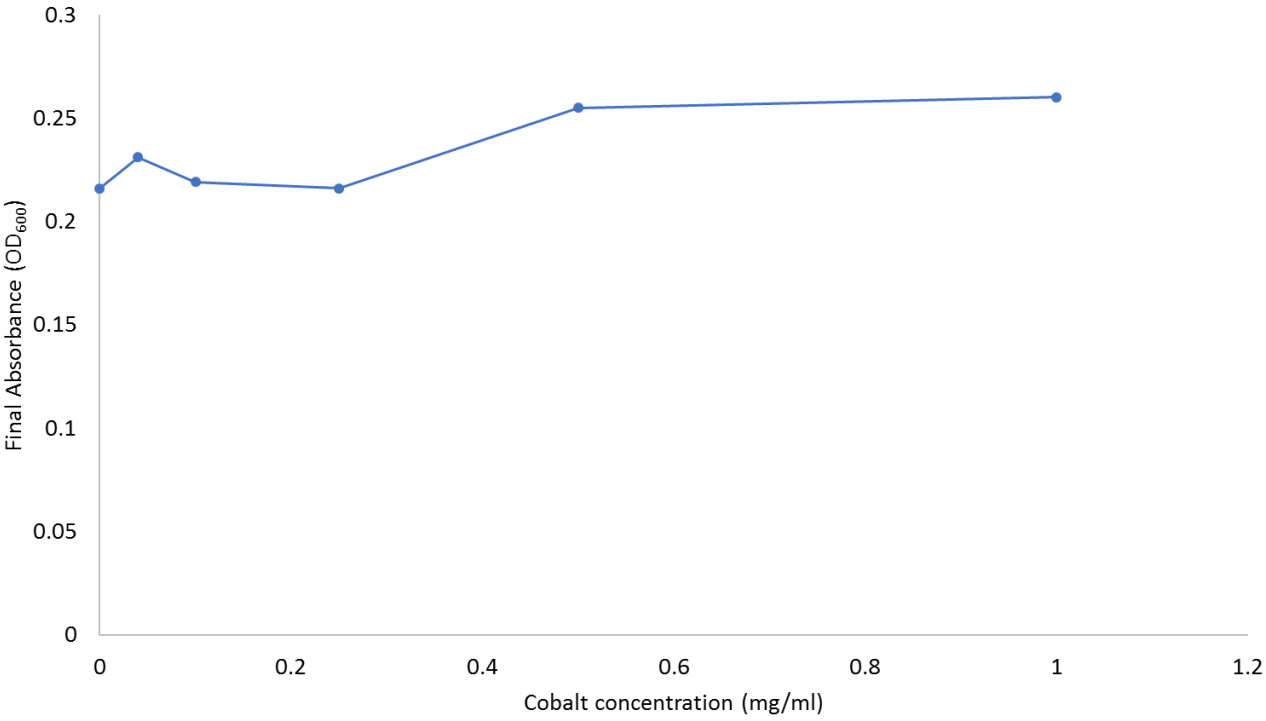


Figure 38 **Cobalt is not toxic for WT *Salmonella*.** Strains were grown in nitrate-sufficient, glycerol-limited M9 minimal media supplemented with cobalt chloride as indicated for 48 hours.

5.3.5 MetE is important for the survival of denitrifying *Salmonella*

Salmonella has the ability to respire on nitrate when oxygen is unavailable, as described in the introduction (Page 30) this results in the net production of N_2O. To create denitrifying conditions *Salmonella* was grown in anaerobic conditions, in M9 minimal media with the addition of 20mM nitrate.

The growth of WT *Salmonella* was compared to *ΔmetE*, *ΔmetH, ΔmetE ΔmetH* and *ΔmetR* when grown in denitrifying conditions and with the addition of vitamin B_{12} or methionine (Figure 39). WT *Salmonella* exhibits similar growth in the absence or presence of methionine, with maximum growth of ≈0.2 OD_{600} reached by 24 hours. The addition of vitamin B_{12} reduces the maximum growth to ≈0.15 OD_{600} which also occurs by 24 hours.

In all three tested conditions the *ΔmetH* mutant grows as per WT. The *ΔmetE* mutant exhibits very little growth until 40 hours at which point a maximum OD of ≈0.15 is reached. If supplemented with either vitamin B_{12} or methionine the growth is comparable to WT.

A double *ΔmetE ΔmetH* mutant has normal growth with the addition of methionine but the addition of vitamin B_{12} does not allow for growth and very little growth is seen for this, and the unsupplemented conditions over 48 hours. There is some growth seen which in both cases may be as a result of cell death and methionine turnover.

When grown with no additional supplements *ΔmetR* exhibits a similar growth pattern to *ΔmetE,* that is minimal growth seen until 40 hours at which point a maximum OD of ≈0.15 is reached. Supplementation with either Vitamin B_{12} or methionine results in a restoration of this growth defect.

The same growth conditions were then carried out but with the additional step of the bottles being saturated with N_2O pre-inoculation. The addition of N_2O to WT *Salmonella* did not result in a growth defect except in the B_{12} supplemented bottles where it further increased the mild growth defect seen. This resulted in a maximum growth of 0.07 OD_{600} by 17 hours which didn't increase by 48 hours. Without the addition of N_2O (but with vitamin B_{12}) the WT strain exhibits similar growth until 17 hours but by 24 hours reaches maximum OD of ≈0.15.

For the *ΔmetE* mutant similar growth is seen to WT with the addition of methionine either with or without exogenous N_2O (Figure 40**Error! Reference source not found.**). While in standard anaerobic conditions the addition of vitamin B_{12} provides a partial restoration of the growth defect when exogenous N_2O is added this affect is greatly reduced and very little growth is seen. In the un-supplemented cultures *ΔmetE* is able to reach an OD comparable to WT growth with methionine by 40 hours. When exogenous N_2O is added however this phenotype is lost and the strain exhibits almost no growth until 48 hours.

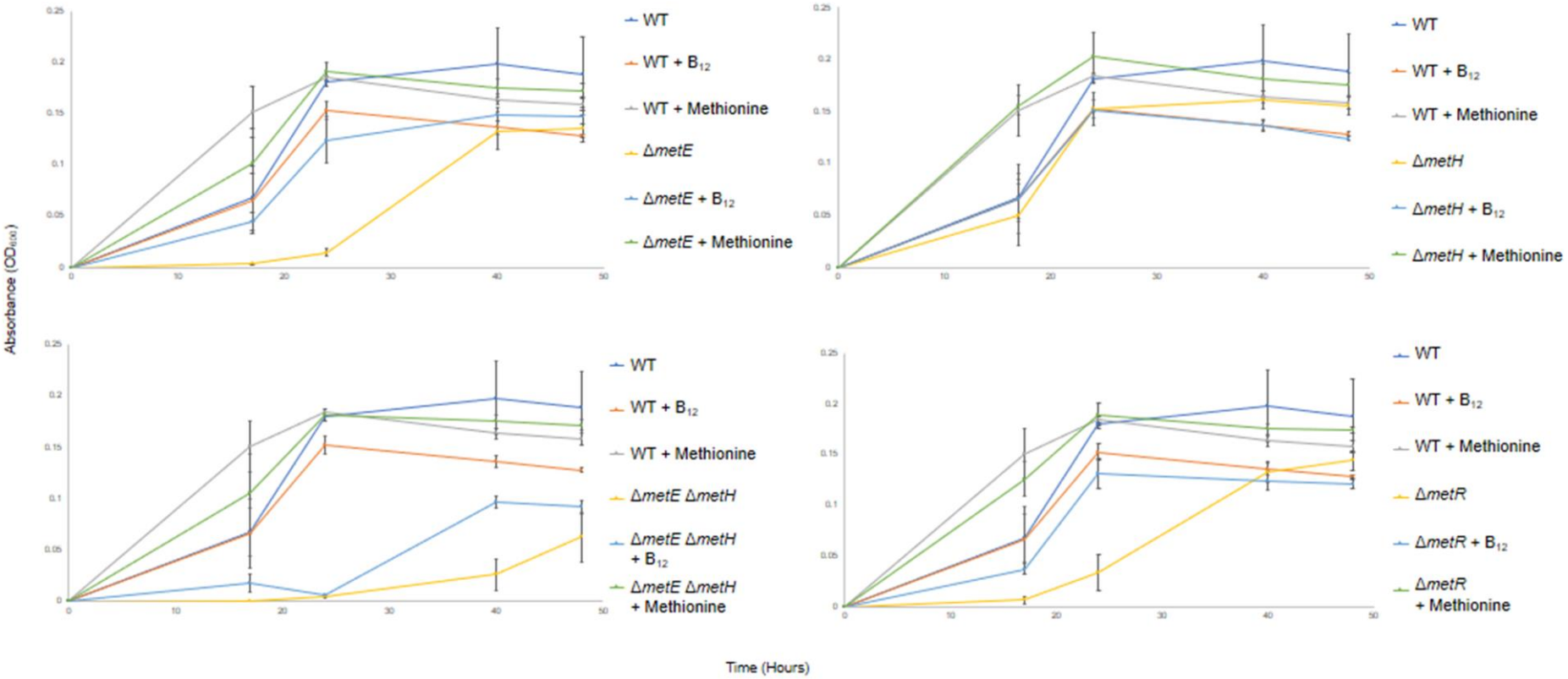


Figure 39 **MetE is important for the survival of denitrifying *Salmonella***. Strains were grown anaerobically in nitrate-sufficient, glycerol-limited M9 minimal media. Where indicated vitamin B_{12} (0.05 µg/ml) or methionine (30 µg/ml) were added. All strains have 3 repeats, and standard error is shown.

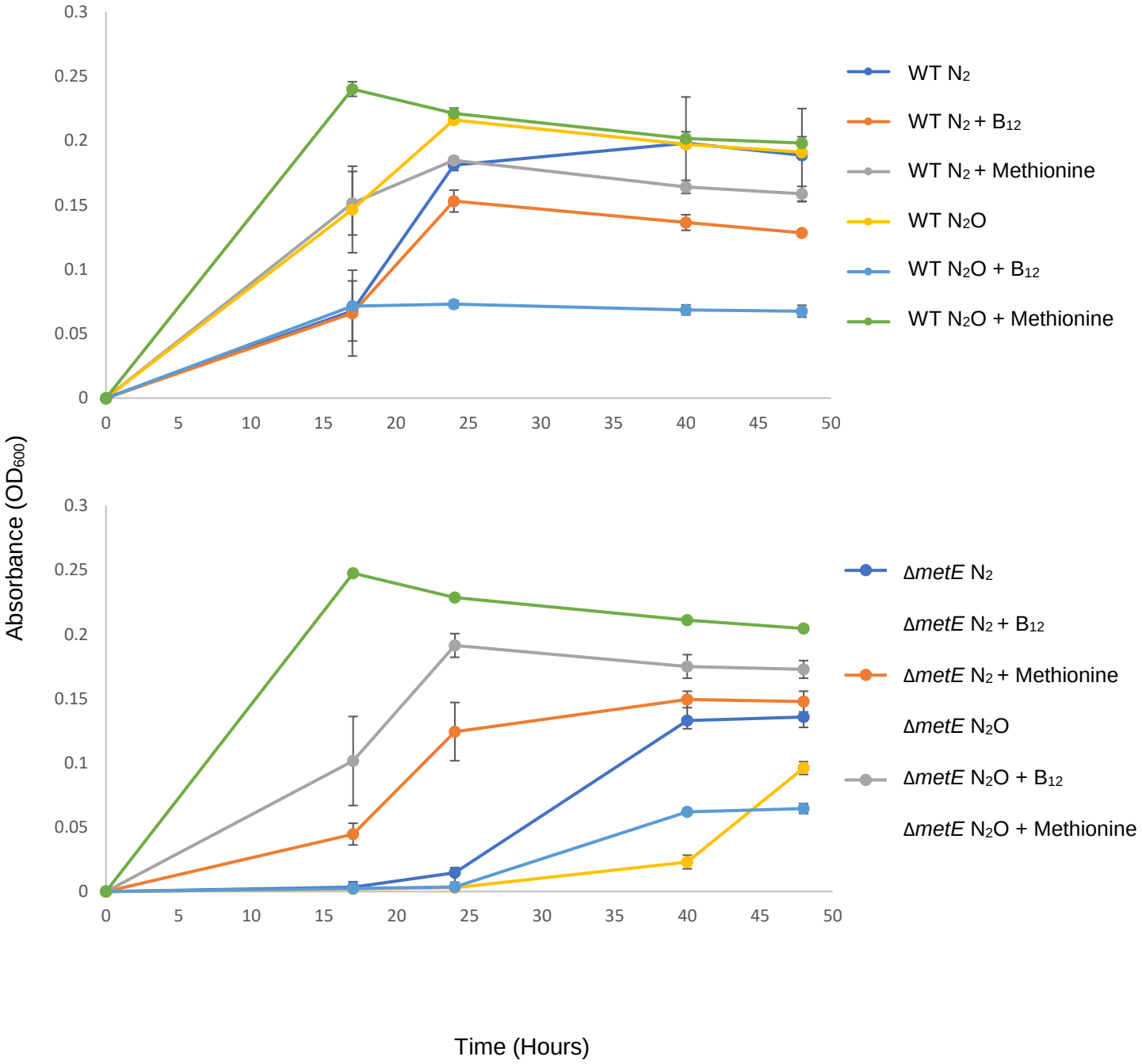


Figure 40 **MetE is important for the survival of *Salmonella* grown with exogenous N_2O**. Strains were grown anaerobically in nitrate-sufficient, glycerol-limited M9 minimal media which had been saturated with N_2O. Where indicated vitamin B_{12} (0.05 µg/ml) or methionine (30 µg/ml) were added. All strains have 3 repeats and SE is shown

5.3.6 Construction of a PocR mutant

A *ΔpocR* deletion mutant, alongside a *ΔmetE ΔpocR* double mutant were created, and correct construction was confirmed by PCR (Figure 41).

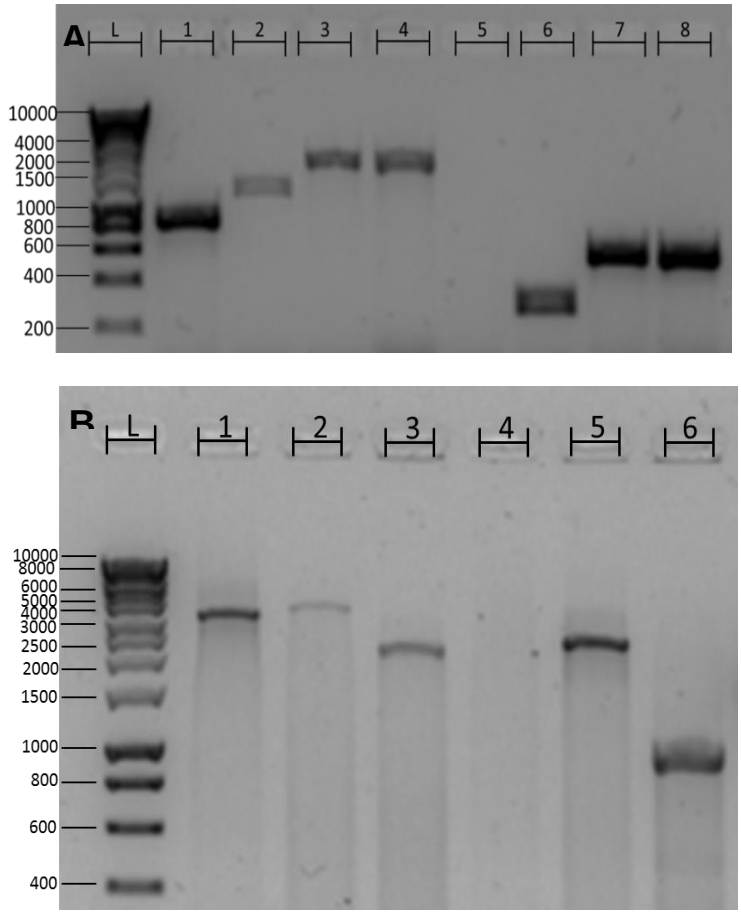


Figure 41 **Confirmation of correct mutant construction of Δ*pocR* and Δ*metE*Δ*pocR* .** Products were run on 1% (w/v) agarose gel against hyperladder II (Bioline). (**A) Verification of *pocR* mutants.** Lanes 1-4: WT (860 bp), Δ*pocR* Cm^r (1395 bp), Δ*pocR* Kan^r (1795 bp), Δ*metE*Δ*pocR* (1795 bp) respectively with *pocR*_Ex_F and *pocR*_Ex_R. Lane 5: WT (no product) with *pocR*_Ex_F and pKD4_R. Lane 6: Δ*pocR* Cm^r (305 bp) with *pocR*_Ex_F and pKD3_R. Lane 7: Δ*pocR* Kan^r (589 bp) with *pocR*_Ex_F and pKD4_R. Lane 8: Δ*metE*Δ*pocR* (589 bp with *pocR*_Ex_F and pKD4_R). **(B) Verification of *metE* mutants.** Lanes 1-3: WT (3054 bp), Δ*metE* (2885 bp), Δ*metE*Δ*pocR* (1654 bp) respectively with *metE*_Ex_F and *metE*_Ex_R. Lane 4-5: WT (no product) and Δ*metE* (1683 bp) respectively with *metE*_Ex_F and pKD4_R. Lane 6: Δ*metE*Δ*pocR* (370 bp) with *metE*_Ex_F and pKD3_R.

5.3.7 PocR is not required for growth in LB or M9 aerobically

Both in LB, and M9 supplemented with glucose, *ΔpocR* exhibits comparable growth to WT. In LB, the double mutant *ΔmetEΔpocR* also grows as WT, but in M9 supplemented with glucose, *ΔmetEΔpocR* grows in the same fashion as *ΔmetE* exhibiting no growth over 24 hours (Figure 42).

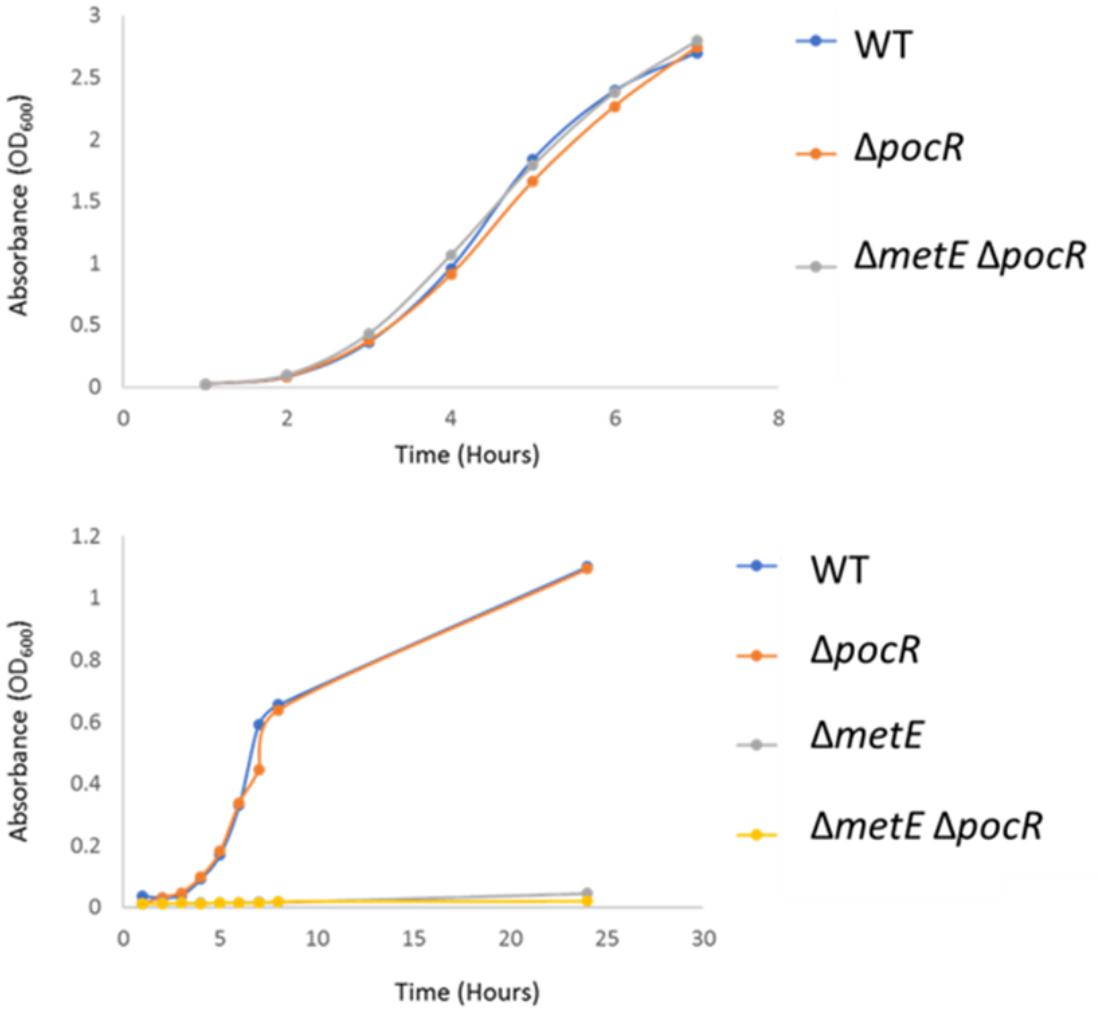


Figure 42 **PocR is not required for growth in LB or M9 aerobically**. Strains were grown in 50ml LB (A), or 50ml M9 + glucose (B).

5.3.8 PocR is not essential for the regulation of *metE*

When growing anaerobically under denitrifying conditions *ΔmetE* struggles to grow but eventually reaches a maximum OD equivalent to WT *Salmonella.* The growth of *ΔmetE ΔpocR* was analysed under these conditions and exhibited a slight restoration in the growth defect in comparison to the single *ΔmetE* strain. That is, the absence of PocR mildly restores the growth defect seen for *ΔmetE.* A single *ΔpocR* mutant has no growth defect under these conditions (Figure 43).

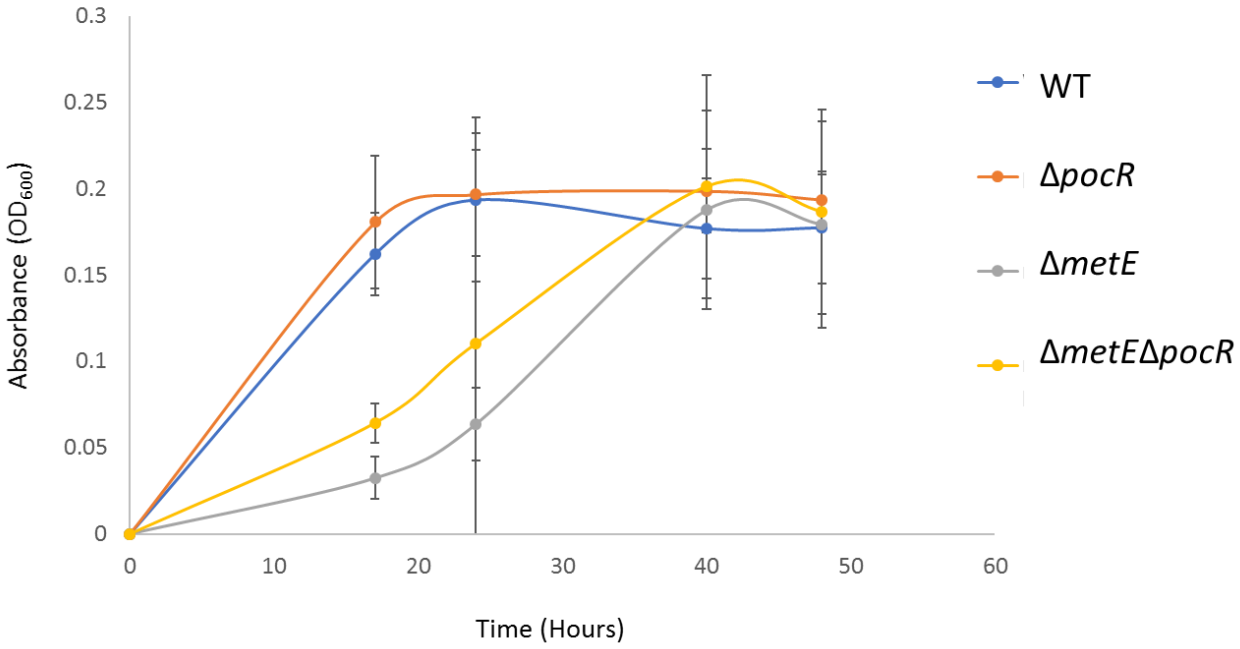


Figure 43 **The growth of Δ*metE* and Δ*metE*Δ*pocR* are comparable when growing under denitrifying conditions.** Strains were grown anaerobically in nitrate-sufficient, glycerol-limited M9 minimal media. All strains have 3 repeats, and standard error is shown.

5.3.9 The *ΔmetE* mutant does not produce the MetE protein

Since a low level of growth is seen for *ΔmetEΔmetH* under anaerobic conditions, without the addition of vitamin B_{12} or methionine, a western blot was performed using an anti-MetE antibody (Mogk et al. 1999, Mordukhova and Pan 2013) to confirm that the protein is not being produced (potentially by a secondary copy of the gene). Samples were taken from anaerobic cultures at various timepoints and at 48hrs for aerobically growing WT. While protein can be seen on the coomassie stain for all samples except *ΔmetE* at 17 hours (when no bacterial growth is seen), MetE is only detectable in the WT sample growing aerobically (Figure 44). Thus, we confirm that no MetE protein is being produced by the *ΔmetE* strain or subsequent double mutants.

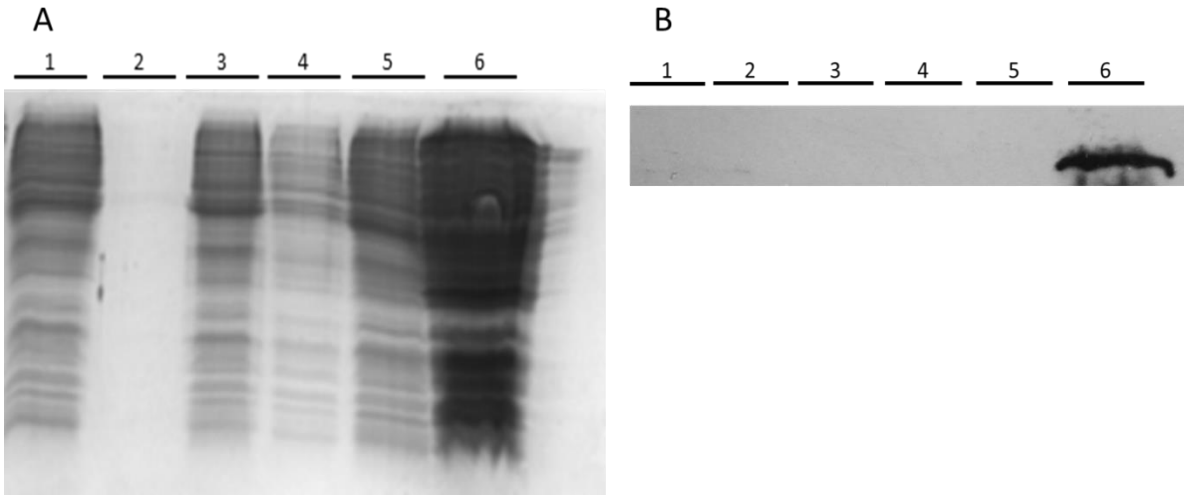


Figure 44 **The MetE protein is not produced by *ΔmetE* or *ΔmetEΔpocR* during anaerobic growth**. Gel and blot lanes: 1) WT 17h. 2) *ΔmetE* 17h. 3) *ΔmetE* 48h. 4) *ΔmetEΔpocR* 17h. 5) *ΔmetEΔpocR* 48h. 6) WT 48h aerobic positive control. (A) Whole cell lysates were run on 12% (v/v) SDS-PAGE. (B) Presence of MetE detected with an anti-MetE rabbit polyclonal antibody.

5.3.10 A *ΔmetE*Δ*cobS* mutant is unable to grow in M9 media in anaerobic conditions

To confirm whether the *ΔmetE* mutant grows anaerobically due to the eventual production of vitamin B_{12} and thus use of MetH, a further double mutant deficient in *metE* and *cobS*, the final gene in the *cob* operon was generated.

It was found that after 48h, *ΔmetE*Δ*cobS* grew significantly less than the WT. These results suggest that this inability to grow is due to the inability to produce vitamin B_{12} (Figure 45).

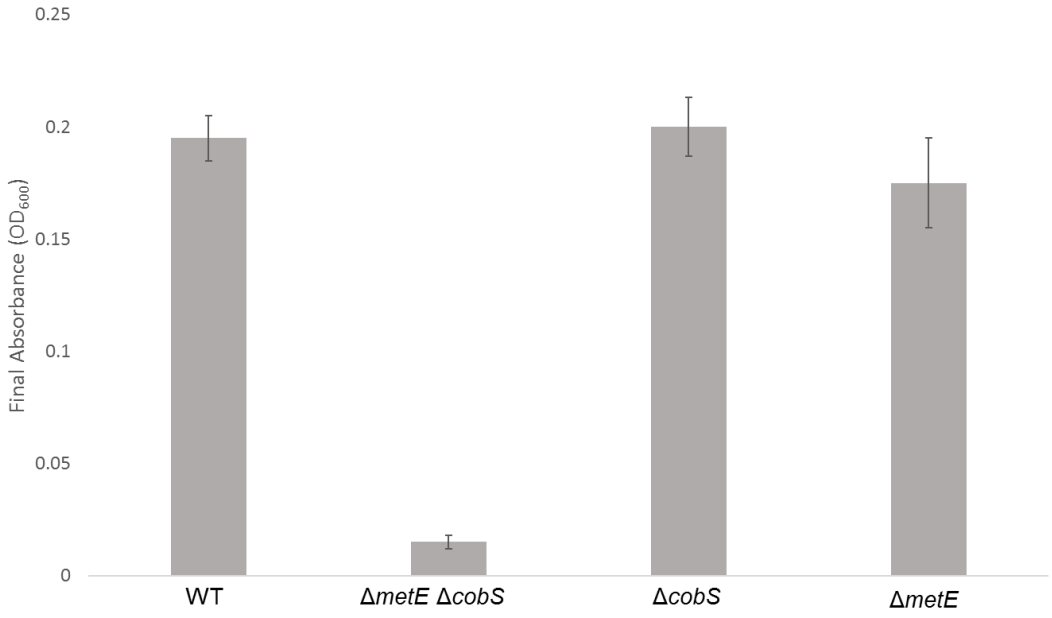


Figure 45 **The deletion of *cobS* and *metE* results in an inability of *S.* Typhimurium to grow.** Δ*metE*Δ*cobS* growth was significantly lower than the WT. Bacteria were grown anaerobically for 48h in M9 minimal medium and OD_{600} measured to determine growth. Data plotted as mean values from one experiment performed in duplicate and error bars representative of SE.

5.3.11 MetE is important for survival of *Salmonella* in macrophages

In order to assess the importance of MetE for the survival of *Salmonella* in macrophages, a gentamycin protection assay was used. This was carried out both in normal DMEM and in DMEM which lacks both methionine and vitamin B_{12}. In normal DMEM media there is no difference between the survival of the three strains tested, WT, *ΔmetE* and *ΔmetH.* However, in the absence of methionine and vitamin B_{12} the *ΔmetE* mutant is significantly ($P > 0.05$) attenuated (Figure 46).

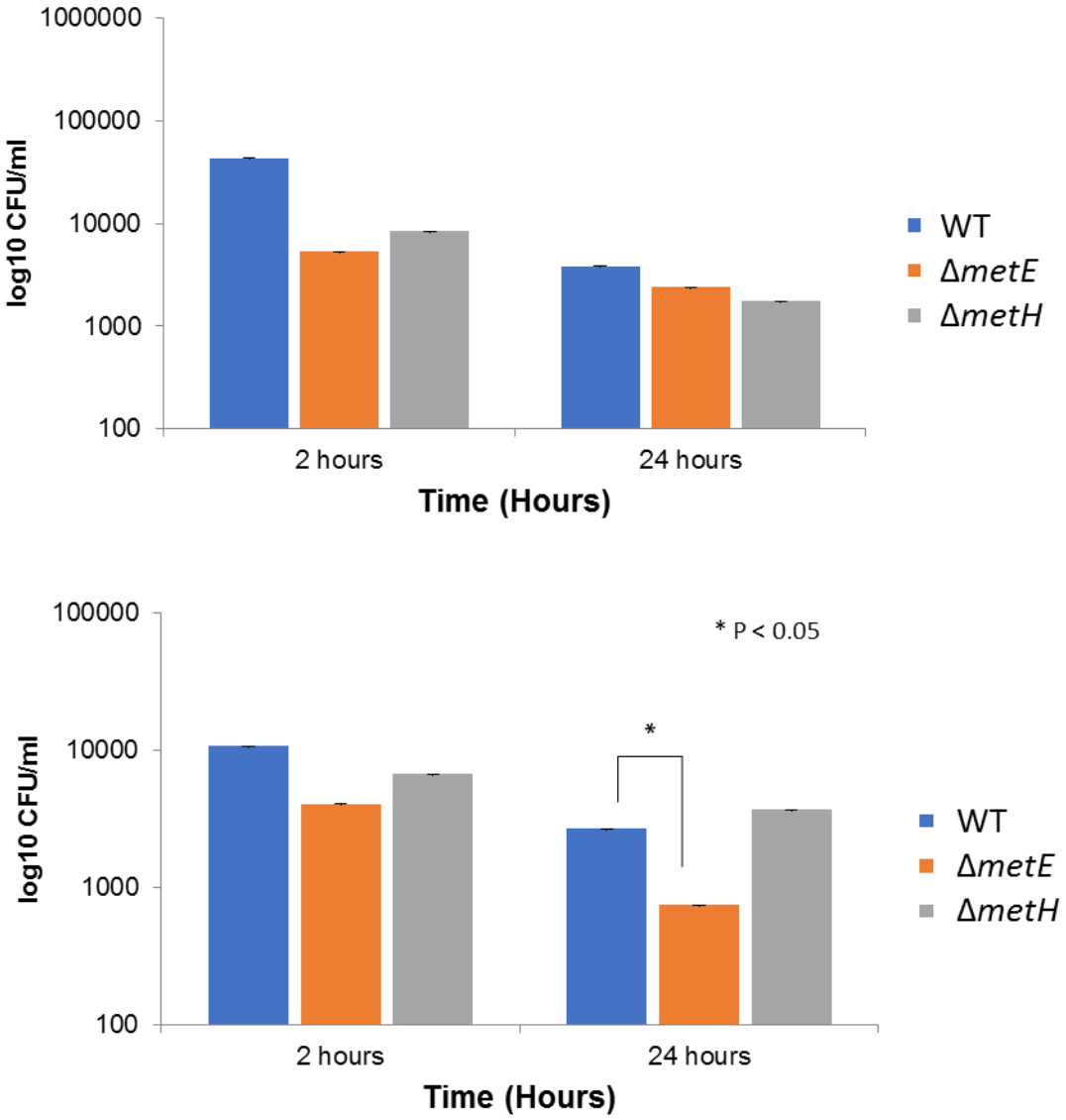


Figure 46 **MetE is required for intracellular survival in murine macrophages in the absence of methionine or vitamin B_{12}.** *Salmonella* uptake into IFN-γ activated macrophages was conducted for 2 hours and 24 hours before gentamicin treatment killed all extracellular bacteria in normal tissue culture media in both the presence and absence of methionine. Internalised bacteria were recovered by lysing the macrophage and the numbers of bacteria enumerated by viable counts. (a) with methionine (b) without methionine/B12. Data shown is an average of three experiments. *Denotes significant difference $P < 0.05$ (independent t-test, n=3).

5.4 Discussion

At the beginning of this PhD there was a clear research gap consisting of two phenomena in *Salmonella*. The first being the inexplicable production of high levels of toxic nitrous oxide, and the second being the highly metabolically expensive synthesis of the seemingly unnecessary compound, vitamin B_{12}. This chapter has made the first steps in explaining these two questions by showing a link between them, and in doing so highlights a potential novel virulence strategy.

The synthesis of methionine by *Salmonella* is essential for growth unless the amino acid is exogenously supplemented. Therefore, a MetH deletion mutant is fully reliant on MetE for this process and a MetE deletion mutant is reliant on MetH. Since MetH requires vitamin B_{12} to produce a functional holoenzyme, a MetE mutant is therefore dependent on the endogenous production of vitamin B_{12} in order to grow. We can conclude that when growth is seen for the MetE mutant strain, or any strains including this deletion in combination with additional mutataions, that conditions are present whereby *Salmonella* is able to synthesise vitamin B_{12} and produce a functioning MetH protein.

The main growth phenotypes of the mutants described in this chapter are summarised in (Table 8). The key mutant is *ΔmetE,* since this strain gives us the most information about the requirements for vitamin B_{12}.

5.4.1 Importance of MetE for denitrifying *Salmonella*

Growth in M9 minimal media with and without the addition of vitamin B_{12} and methionine was used to assess the survival of *Salmonella*'s requirements and synthesis of vitamin B_{12} aerobically. WT *Salmonella,* as well as *ΔmetH* are able to use the functional vitamin B_{12} independent MetE protein in order to synthesise methionine and therefore exhibit the same levels of growth in the presence and absence of vitamin B_{12} or methionine. The *ΔmetE* mutant is unable to grow in these conditions without the addition of B_{12} or methionine – this confirms that *Salmonella* is unable to aerobically synthesise vitamin B_{12}. The same phenotype is also seen for *ΔmetR,* as a positive regulator of both MetE and MetH this shows that aerobically

Table 8: **Summary of growth abilities of *Salmonella* mutants.** A tick (✓) indicated the strain grew as WT, a cross (✕) indicates no growth was seen, both (✕/✓) indicates growth lower than that seen for WT.

	LB	M9 aerobic			M9 anaerobic		
			+ B_{12}	+ Methionine		+ B_{12}	+ Methionine
WT	✓	✓	✓	✓	✓	✓	✓
ΔmetE	✓	✕	✓	✓	✕/✓	✓	✓
ΔmetH	✓	✓	✓	✓	✓	✓	✓
ΔmetE ΔmetH	✓	✕	✕	✓	✕	✕/✓	✓
ΔmetR	✓	✕	✓	✓	✕/✓	✓	✓
ΔpocR	✓	✓			✕/✓		
ΔmetE ΔpocR	✓	✕			✕/✓		

Salmonella is reliant on MetR in order to activate either the MetE or MetH protein. This phenotype can also be recovered by the addition of vitamin $B_{12,}$ therefore the conclusion can be drawn that vitamin B_{12} itself is able to regulate either the MetE or MetH protein (most logically MetH). When both MetE and MetH are absent, *Salmonella* is only able to grow aerobically on M9 with the addition of methionine.

Conditions where *Salmonella* will be required to use nitrate or nitrate for the terminal electron acceptor in respiration can occur outside the host but also in the mammalian intestine, as part of the host-generated inflammatory response (Lopez et al, 2012). Growth of the various mutants was therefore analysed on M9 minimal media anaerobically with nitrate present to assess the importance of these genes while undergoing denitrification, which can occur when *Salmonella* is residing in the intestine. WT *Salmonella* grows the same with and without the addition of methionine, so while it only is able to reach a maximum OD_{600} of ≈0.2, this is probably due to the minimal media and anaerobic conditions in general and not to do with vitamin B_{12}-/methionine metabolism. Similarly to aerobic growth on M9, *ΔmetH* is able to grow like WT in all conditions, this confirms that the MetE protein is produced and functional, to provide the bacteria with enough methionine for growth. While for the first 24 hours of growth *ΔmetE* shows very little growth, by 40 hours the strain is able to reach an OD comparable to WT. Since the *ΔmetE* strain relies on MetH for its methionine synthesis, and MetH is dependent on vitamin $B_{12,}$ in these conditions, for this strain to grow, *Salmonella* needs to be synthesising enough vitamin B_{12} in order for growth to be seen. It therefore is likely to be the case that during the first 24 hours of growth the bacteria is slowly attempting to build up a vitamin B_{12} pool, however since growth is also reliant on denitrification there will be a net release of nitrous oxide which will be interacting with the vitamin. Therefore, in order for visible growth to be seen for *ΔmetE* a point must be reached where more vitamin B_{12} is being produced than is being targeted by nitrous oxide.

A similar growth pattern is seen for *ΔmetR* as for *ΔmetE;*growth is slow until 40 hours when growth can be seen, and the growth defect can be restored by the addition of either vitamin B_{12} or methionine. The *ΔmetR* mutant has a functional MetH and MetE – and while MetH will rely on vitamin $B_{12,}$ MetE should be able to produce methionine from the beginning of growth such as occurs for *ΔmetH.* This phenotype therefore

suggests that the activation of MetE is dependent on either MetR or on vitamin B_{12}, since at later time points the vitamin B_{12} pool should have been established.

This hypothesis for the phenotype of *ΔmetE* and *ΔmetR* is corroborated by the growth phenotype of *ΔmetE* Δ*cobS* under the same conditions. CobS is one of the final genes in the *cob* operon and is its deletion is known to stop the synthesis of vitamin B_{12} in *Salmonella.* This strain shows nearly no growth over 48 hours.

From the previous results it would be expected that a *ΔmetE ΔmetH* double mutant would be unable to grow without the addition of methionine. However, growth, albeit very low, is seen for both the unsupplemented culture and that supplemented with vitamin B_{12}. There are two potential explanations for this observation, the first being that after 24 hours there is a low level of methionine turnover resulting from cell death from the initial inoculation. The second possibility is that by 40/48 hours there is contamination in the cultures, as described (Page 49), each timepoint requires four needles, two one-use needles for liquid sampling, and two multi use needles for gas sampling to be inserted through the silicon lid. While precautions were taken such as cleaning the outside of the silicon lid with ethanol, and cleaning the reusable Hamilton gas syringe between sampling, this is not a totally sterile process. Samples could be taken throughout to determine if contamination is present.

5.4.2 Vitamin B_{12} toxicity

Vitamin B_{12} was initially added to aerobic M9 cultures at 100 µg/ml as per (Sullivan et al. 2013) where it only had a slight negative effect on the growth of WT *Salmonella*. However, when this same concentration was added to M9 anaerobically, a significant growth defect was seen, reducing the OD of the WT to that similar to non-supplemented *Salmonella*. A concentration of vitamin B_{12} was determined where *ΔmetE* and WT *Salmonella* grew comparably, this was 0.05 µg/ml.

There is no information in the literature about a toxic effect of vitamin B_{12} on *Salmonella,* or in fact for any bacteria. In humans no toxicity has been observed for vitamin B_{12} and no safe upper limits have been defined (Flynn et al. 2003, Burgess, Smid and van Sinderen 2009). There is a small body of work looking at the toxicity of vitamin B_{12} on mammalian cells due to catalysing the oxidation of sulfhydryl groups in

sulfhydryl-dependent cells (Toohey 1975); this however should not have an effect on *Salmonella* in anaerobic conditions, so is unlikely to be the explanation.

It was however considered that the toxicity could be due to the cobalt contained within the compound. However even at levels far exceeding those which result in a toxic effect from vitamin B_{12}, no toxicity was seen and hence it is concluded that the toxicity seen when *Salmonella* SL1344 WT is grown in the presence of vitamin B_{12} is not due to cobalt toxicity.

Another possibility is that vitamin B_{12} has a regulatory effect on *Salmonella*. MetE is known to be down-regulated by MetJ as well as the MetH-B_{12} holoenzyme (Wu, Urbanowski and Stauffer 1992). Therefore, in conditions with high levels of vitamin B_{12} it could be expected that MetE is downregulated. However, this would be reliant on the MetH-B_{12} holoenzyme and so growth should not be affected.

It should be noted that high levels of vitamin B_{12} are unlikely to occur in natural conditions, since the synthesis of the vitamin is so metabolically expensive it is highly improbable that any bacteria, even on lysis, would be releasing the compound in any significant concentrations. The recommended daily intake for humans varies for different age groups but is ≈2 µg, it can be assumed therefore that concentrations would never reach 100 µg/ml in the intestines or anywhere that *Salmonella* might reside. The toxic effect could therefore be simply a result of such an unusually high level of a very large molecule.

The possibly was considered that even though a *metE* knockout was constructed the bacteria was still producing the MetE protein via a potential secondary copy of the gene, and this was allowing for the eventual growth of a *ΔmetE ΔmetH* mutant, or indeed the single *ΔmetE* strain. To confirm this was not the case a western blot was carried out using an anti-MetE antibody

A further interesting observation was made during the Western blot analysis. No MetE protein was detected from the WT strain at the 17h time-point, suggesting it is only relying on MetH for methionine synthesis. Potentially, the MetE protein is used at later stages of growth, when N_2O levels have begun to accumulate and inactivate vitamin B12. However, this hypothesis highlights a difference in comparison to the anaerobic growth curve data and does not explain why the growth of *ΔmetE* was reduced in comparison to the WT at the 17h time-point if both strains are using the same enzyme

for synthesis of methionine, not that WT is using MetE as suspected. Potentially, MetE may be present in the WT sample but at a lower concentration than the aerobic sample. Exposure time may need to be increased during blot visualisation in order to detect low levels of the protein. Further investigation should be undertaken in which later time-points are sampled to quantify MetE production by the anaerobic WT strain.

5.4.3 Regulation of the *cob* operon by PocR

A PocR mutant, and subsequent *ΔmetEΔpocR* strain were constructed to determine the importance of this gene for *Salmonella*, specifically in the regulation of the *cob* operon. Since the *cob* operon is so large and the synthesis of vitamin B_{12} so metabolically expensive it is logical to assume there is efficient regulation.

The *cob* operon is transcribed adjacently to the *pdu* operon, which encodes genes required for the vitamin B_{12} dependent utilisation of propanediol as an energy source; both operons are under the transcriptional control of PocR, encoded by *pocR* which is situated between the two operons (Ailion et al. 1993). Aerobically it is known that Crp/cAMP are important for induction of PocR and therefore the two operons, and anaerobically ArcA/ArcB are also required. The importance of Crp suggests that carbon utilisation is a primarly function of vitamin B_{12}. Additionally it has been shown that 1,2-propanediol enhances the binding of PocR and as such increases the activation of the *cob* operon – presumably to provide the vitamin B_{12} required for the utilisation of this energy source (Rondon and Escalante-Semerena 1996). A study has also shown that PocR is induced by glycerol – the carbon source used for anaerobic growth in this study (Bobik et al. 1992). However there lies a paradox in this co-regulation. Aerobically *Salmonella* is unable to produce vitamin B_{12} (even if the *cob* operon is fully transcribed) and therefore propanediol cannot be utilised. Anaerobically, whilst the *pdu* operon is induced, oxygen is required for the utilisation of the carbon source, alternative electron acceptors cannot be used and propanediol cannot be fermented. Therefore, while it has been hypothesised in the literature that the fact that PocR is the major regulator of the *cob* operon suggests propanediol respiration is the primary use of vitamin B_{12} in *Salmonella*, this seems unlikely to be the case (Bobik et al. 1992).

Another candidate regulator of the *cob* operon, CsrA (carbon storage regulator A), has been proposed. First identified in *E. coli*, CsrA is classed as a global regulator, and until recently in *Salmonella* was known only to upregulate genes of the pathogenicity island SPI-1, necessary for intracellular invasion (Lawhon et al. 2003, Altier, Suyemoto and Lawhon 2000). However, Lawhon et al. (2003) identified that genes of the *cob* operon were significantly downregulated in a Δ*csrA* mutant in comparison to the WT. Furthermore a downregulation of genes located in the *eut* and *pdu* operons, both of which are dependent on the presence of B_{12}, were also observed, signifying that CsrA has multiple roles aside from SPI-1 regulation (Lawhon et al. 2003).

There are many possibilities for future investigations into the regulation of vitamin B_{12} synthesis by *Salmonella*. Primarily it would be interesting to investigate the anaerobic growth of a triple deletion mutant in *metE, pocR* and *csrA.* If this mutant was unable to grow anaerobically, it would indicate that either *pocR* or *csrA* are essential to B_{12} synthesis and exhibit functional overlap. However, if growth was still observed, it would show that there are other, unknown regulators yet to be identified.

One study looking at the transcription levels of the *cob* operon under different conditions highlights the 40 fold increase in transcript levels between the anaerobic fermentation of glucose and under conditions of anaerobic respiration. It is stated that they believe there to be unidentified functions which exist that require B_{12} under conditions of anaerobic respiration but that since *cob* mutants are able to grow normally on glycerol fumarate they believe them to be inessential (Escalante-Semerena and Roth 1987). In the context of this study it is not surprising that *cob* mutants struggled to grow on glycerol fumarate – and it is more likely that the operon is being upregulated in anaerobic conditions for growth of *Salmonella* on nitrate.

The results in this study suggest that the absence of PocR partially restores the growth phenotype of *ΔmetE* when growing anaerobically on nitrate. This therefore indicates that there is an alternative regulatory mechanism inducing the expression of the *cob* operon for B_{12} production, enabling *Salmonella* to continue to use MetH to produce methionine.

One potential explanation for this phenotype is that there are multiple regulators inducing expression of the *cob* operon in the absence of PocR, resulting in a greater

level of transcription of *cob* genes. This would account for the increased growth rate of *ΔmetEΔpocR* compared to *ΔmetE.*

Figure 47 outlines a possible model to explain the results observed in this study. The binding of PocR to its recognition site potentially could block the binding sites of other, currently unknown transcription factors of the cob operon. Whereas, in the absence of PocR these sites would be available.

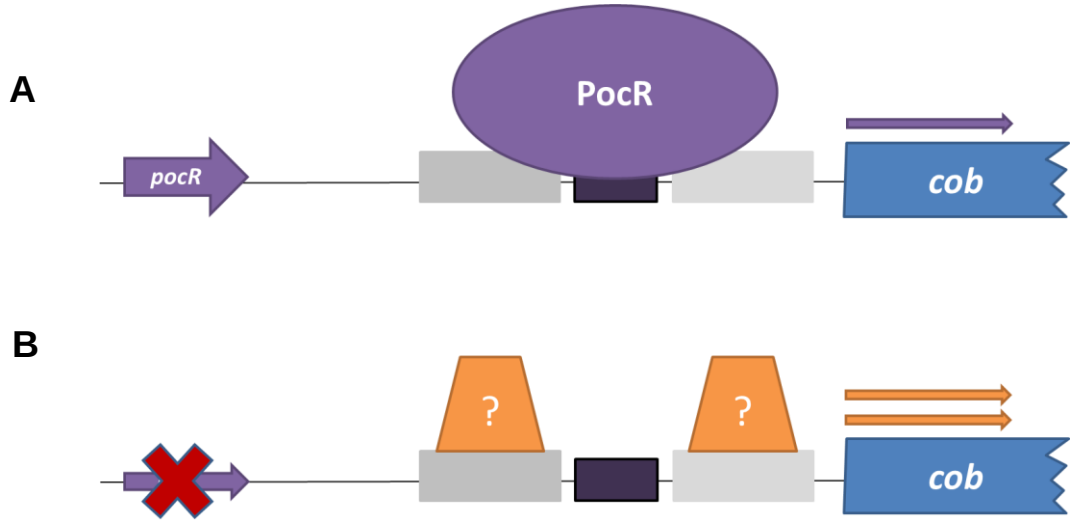


Figure 47 **Working model – regulation of the *cob* operon** (A) **Proposed model for induction of the *cob* operon by PocR**. When transcribed, PocR binds to its binding site (dark purple box) and induces *cob* expression. Access to other transcription factor binding sites (grey boxes) is blocked by the presence of PocR. (B) **The proposed mechanism for the positive regulation of the *cob* operon in the absence of PocR**. Binding sites for other transcription factors are accessible and interaction may induce expression of the *cob* operon.

5.4.4 The pathogenic effect of the production of nitrous oxide by *Salmonella* in the intestine

Based on the results presented in this chapter the hypothesis was drawn that there is a distinct link between the two phenomena highlighted in the introduction to this chapter – the production and release of large quantities of nitrous oxide and the synthesis of the metabolically expensive vitamin B_{12}.

This study has shown that *Salmonella* is afforded a growth benefit by MetE when growing anaerobically on nitrate, that is, that the absence of this gene results in a distinct growth defect.

Nitrate is present in the human intestine from two primary routes, the first being dietry products and the second endogenous synthesis. Different diets result in differing amounts of nitrate in the gut, with highest concentrations coming from nitrate-rich vegetables, particularly leafy greens (Lidder and Webb 2013). Vegetarians therefore on average have higher daily intakes of nitrate, at ~4.3 mM nitrate/day compared to ~1.2 mM nitrate for a standard western diet, the Acceptable Daily Intake has been set at 5 mM nitrate/day since 1962 (Katan 2009).

While most early studies looking at nitrate in the gastrointestinal tract are focused on this from a human cell perspective, specifically in relation to cancer, these studies give some insights into the nitrate environment of the gut.

A study looking at the nitrate and nitrite concentrations in human fecal and ileostomy (a method of sampling directly from the end of the small intestine) samples concluded that the lower gastrointestinal tract favours denitrification over nitritrication. Fecal nitrate levels ranged from 0 to 14 µmol/kg (0 to 0.9 ppm) and ileostomy samples from 0 to 7 µmol/kg (0 to 0.4 ppm). Experiments were also carried out that showed that the addition of 500 µmol/kg nitrate to a fecal sample resulted in this nitrate being reduced 10 fold within 15 minutes and fully depleted within 2 hours; thus there must be high levels of denitryfing microorganisms present in faeces and additionally suggesting the high turnover of nitrate in the gut may result in false underreporting of nitrate concentrations from sampling (Saul et al. 1981).

It has been known that there is endogenous production of nitrate in the human intestine since at least 1978 (Tannenbaum et al. 1978) but more modern studies have looked at this from the perspective of gut bacteria.

Pathogenic bacteria, including *Salmonella*, can induce an inflammatory response in the intestine. Whilst this was traditionally thought to be detrimental to the pathogen, since it is part of the host inflammatory response, more recently this has be shown to in fact be beneficial to pathogens, and that triggering the host immune's defence can disrupt the gut environment beneficially for pathogenic bacteria (Stecher et al. 2007).

This can result in the release of nitrate into the gut lumen, and additionally in the decrease in the representation of obligate anaerobes and an increase in facultative anaerobic members of the Enterobacteriaceae family (Winter et al. 2013, Lupp et al. 2007). In fact, it has been shown that in order to outcompete the microbiota and cause an infection, *Salmonella* is reliant on its ability to trigger inflammation. The T3SS-1 effector protein, SopE, enhances intestinal inflammation during *Salmonella* infection, via the activation of caspase 1 and the subsequent cleavage of interleukins into active cytokines (Hoffmann et al. 2010, Lopez et al. 2012). These cytokines, IL-1β and IL-18 contribute to the production of IFN-γ which stimulates iNOS to produce NO. Nitric oxide can then react with ROS and produce peroxynitrite, $ONOO^-$, which isomerises to nitrate, NO_3 (Szabó, Ischiropoulos and Radi 2007). This means that SopE-dependent iNOS expression generates host-derived nitrate and this nitrate has been shown to increase the growth of *Salmonella* (Lopez et al. 2012). Interestingly, nitrate is shown to be a negative regulator of tetrathionate respiration. Potentially this shows that nitrate respiration is preferred to tetrathionate respiration in the intestine (Lopez et al. 2012).

Similarly to the pathogen mediated host release production of nitrate, *Salmonella* also triggers the host to produce tetrathionate, which can then be used as an alternative electron acceptor (Hensel et al. 1999). This occurs via the reaction of ROS generated by inflammation with endogenous luminal thiosulfate. Indeed, data shows that the ability to respire tetrathionate provides *Salmonella* a distinct advantage when growing in the inflamed gut (Winter et al. 2010, Winter and Bäumler 2011). This therefore is a secondary example of *Salmonella* exploiting the host immune response to provide an altered ecological niche beneficial for its growth. Most interestingly however is the carbon sources which are required to allow for tetrathionate respiration, either ethanolamine or propanediol, both of which require vitamin B_{12}. It is noted by the

authors that tetrathionate respiration using ethanolamine or propanediol are the only conditions they know under which wild-type *Salmonella* requires B_{12} for growth (Price-Carter et al. 2001).

Stecher *et al.,* propose a working model for the three-way microbiota-pathogen-host interaction during murine *Salmonella* colitis, which suggests that *Salmonella* infection involves both triggering inflammation and secondarily surviving and thriving from the change in the ecological niche.

The results of this chapter contribute additional knowledge to this story, by showing that not only does the ability to respire on nitrate mean that *Salmonella* can survive within the inflamed intestine but that in doing so will release toxic nitrous oxide to further target the microbiota and potentially the host itself.

The importance of the gut microbiota is becoming ever more diverse, but one of the well known functions of these bacteria is in colonisation resistance, via the barrier effect. This happens in three main ways, the first being the commensal bacteria providing a physical barrier to the pathogen, which aim to target attachment and subsequent invasion into epithelial cells, the second involves a symbiotic relationship with the host to control nutrient availability and the third in activity producing antimicrobial compounds (Guarner and Malagelada 2003, Buffie and Pamer 2013).

It is therefore of interest to consider the impact of nitrous oxide on the commensals. The four dominant phyla in the human gut are Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria, while each individual's gut microbiota is different there are some species which are common to most. These include, *Bacteroides fragilis, Bacteroides melaninogenicus, Bacteroides oralis, Bifidobacterium bifidum Enterococcus faecalis, Escherichia coli, Enterobacter sp., Klebsiella sp.* and *Lactobacillus.* There are three processes to assess here, the vitamin B_{12} independent synthesis of methionine, demonstrated by the presence of MetE, the ability to synthesise vitamin B_{12} *de novo* and the ability to reduce nitrous oxide to N_2 via the enzyme NosZ (Table 9). Of all the commensal bacteria analysed, none have the ability to reduce N_2O – and therefore the presence of this gas in high volumes in the intestine will impact on the vitamin B_{12} pool and any vitamin B_{12} they are able to synthesise. Only one species however, *Lactobacillus*, is able to synthesise vitamin B_{12} *de novo* and we can therefore conclude all other rely on vitamin B_{12} uptake from the

environment – of course as the vitamin B_{12} pool is depleted this will become a limited resource. Lastly, the presence of the vitamin B_{12} independent methionine synthase was determined, any bacteria lacking this strain will rely on the vitamin B_{12} dependent MetH in order to synthesise methionine, only some members of the *Klebsiella* species and some strains of *Escherichia coli* have *metE*.

Table 9 Presence of *metE, nosZ* and ability to synthesise B_{12} of eight common commensal bacteria and human epithelial cells

	metE	B_{12} synthesis	*nosZ*
Bacteroides fragilis	✕	✕	✕
Bacteroides melaninogenicus	✕	✕	✕
Bacteroides oralis	✕	✕	✕
Bifidobacterium bifidum	✕	✕	✕
Enterococcus faecalis	✕	✕	✕
Escherichia coli	✓	✕	✕
Klebsiella sp	✓	✕	✕
Lactobacillus sp	✕	✓	✕
Human epithelial cells	✕	✕	✕

Therefore, the overriding model drawn from this data is that when residing in the intestine *Salmonella* triggers the release of nitrate into the gut lumen and uses this for denitrification. This then results in previously inexplicably high levels of nitrous oxide. The nitrous oxide released will target the vitamin B_{12} pool and as such the commensals which rely on this cofactor for multiple processes, including methionine synthesis, will not be able to survive. Additionally, *Salmonella* may be able to 'anaesthetise' the host epithelium as similarly to the majority of commensal bacteria these cells are reliant on vitamin B_{12} and lack the ability to produce it. The working model produced by Stecher *et al.,* has been adapted to include this hypothesis (Figure 48) (Stecher et al. 2007).

The ability of *Salmonella* to survival and reproduce inside macrophages is of key importance to its pathogenesis in causing systemic infections. The importance of MetE in macrophage survival was shown when media was lacking in methionine. This therefore suggests that methionine is not present in macrophages, but additionally that nitric oxide, which is produced by macrophages, is also involved in the targeting of the vitamin B_{12} pool. As discussed at more length in Chapter 3, genes which are important for intracellular survival of *Salmonella* inside macrophages have the potential to be further studied in relation to making a new vaccine strain.

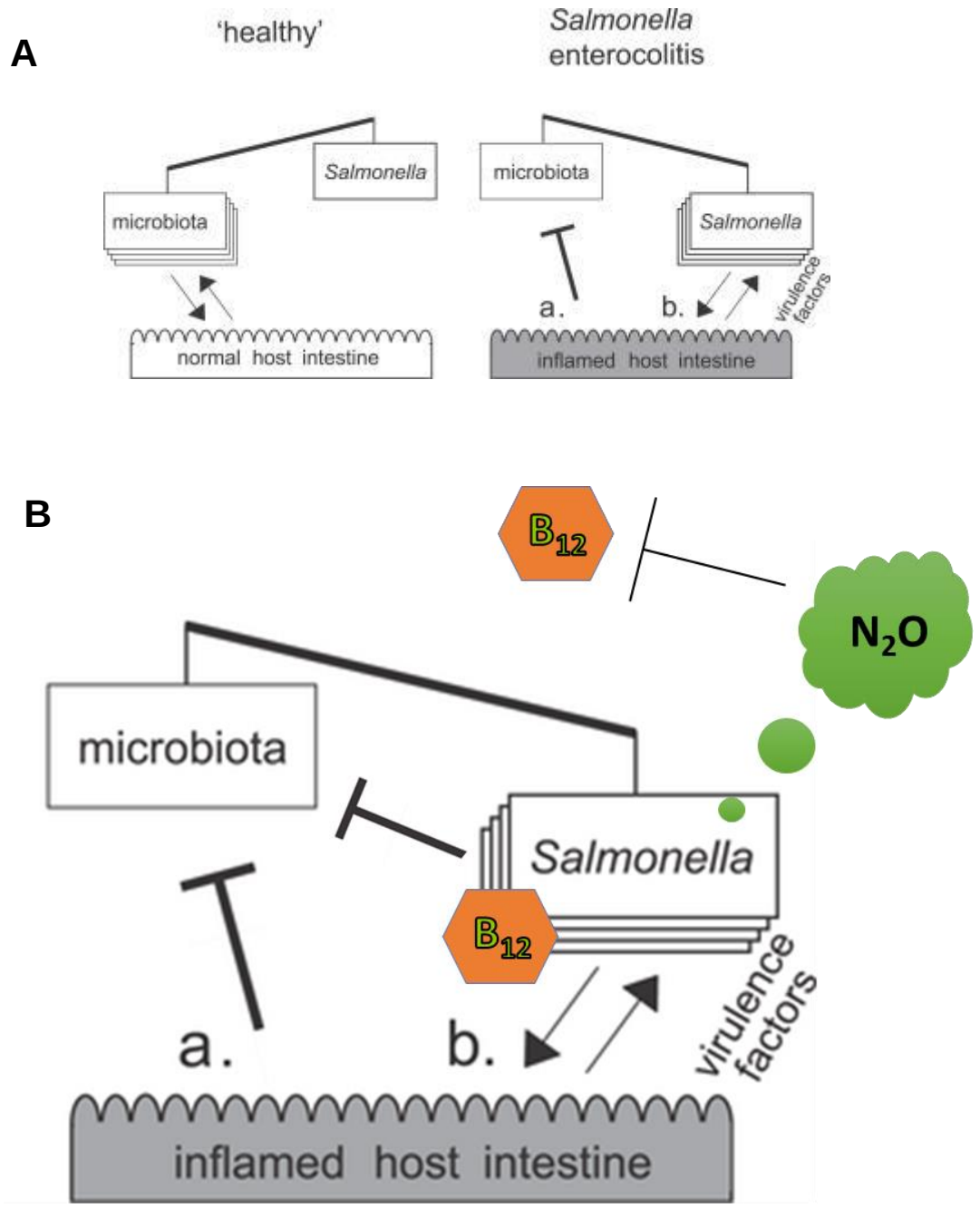


Figure 48 **Working model** A) Working model adapted from (Stecher et al. 2007) showing the three way interactions between the host, microbiota and *Salmonella* in normal conditions and in enterocolitis. B) Altered model of *Salmonella* enterocolitis with the addition of N_2O release by *Salmonella* targeting the vitamin B_{12} pool and thus the inhibition of the microbiota by the pathogen.

5.4.5 Future work

Further work should now be done to show the importance of this system in competition with the gut microbiota and in the survival and pathogenesis of *Salmonella in vivo.* Initially simple cultured competition experiments could be conducted, mimicking the previous experiments under denitrifying conditions, but with the addition of one or multiple commensal bacteria. To create a more realistic environment, a microfluidic gut model could be used. Microfluidic model organs are increasingly being used for many different functions as they enable a much higher level of control over conditions than in animal models (Esch, Bahinski and Huh 2015, Bhatia and Ingber 2014, Huh, Hamilton and Ingber 2011). While new in the gut microbiota field there are a number of systems being used, these allow a defined 'microbiota' to be stabilised before the addition of the pathogen (Shah et al. 2016, Kim and Ingber 2013, Kim et al. 2016b). Various mammalian cells can also be cultured in the system and so one could quantify the ability of *Salmonella* to cross over the epithelium, and potentially invade macrophages.

If these experiments, potentially in combination with mouse model studies, confirm the importance of nitrous oxide production, vitamin B_{12} synthesis and the ability to produce methionine independently of vitamin B_{12} for *Salmonella* in outcoming the gut commensals, this is an exciting new window of research.

The most logical reaction would be to either attempt to reduce the nitrous oxide produced by *Salmonella,* or reduce its detrimental effects on the gut commensals. This could be done by introducing bacteria capable of complete denitrification to the gut. Another option would be in the creation of 'Smart' probiotics, that is, genetically modified probiotic bacteria. If our hypothesis is correct, *Salmonella* is able to exploit the fact that the common gut microbiota are both unable to reduce nitrous oxide and unable to synthesise essential methionine in its presence. Therefore, if these bacteria were altered to either produce NosZ to reduce N_2O or MetE, to enable them to synthesise methionine independently of vitamin B_{12} this might lose *Salmonella* one of its pieces of pathogenic machinery.

6 A correlation between nitrous oxide emissions and vitamin B_{12} synthesis in *Salmonella* serovars

6.1 Introduction

Different *Salmonella* serovars are distinguished by the somatic (O), flagellar (H) and capsular (Vi) antigens on the outer membrane (Shi et al. 2015). As introduced previously (Page 18) different serovars infect different hosts and cause different disease types, so naturally, in order to do so, there are differences in the genes they have retained and in their nutritional requirements. One difference we have noticed is the lack of conservation in the *cob* operon between different serovars, resulting in differences in their ability to synthesise vitamin B_{12}. As described in the introduction (Page 38), the synthesis of vitamin B_{12} is split into three sections. Mutations, or loss of function, can therefore occur in different parts of the operon resulting in different effects, for example, a strain lacking a complete Part I can synthesise cobalamin if provided with cobinamide and Part II of the *cob* operon is still able to synthesise vitamin B_{12} if supplied with dimethylbenzimidazole (DMB) (Roth et al. 1993).

6.1.1 Salmonella Typhimurium SL1344/474

S. enterica serovar Typhimurium strains are split into sequence types (STs), both 4/74 and SL1344 belong to ST19 (Kingsley et al. 2009). *Salmonella* Typhimurium 4/74 was orginally isolated from a calf exhibiting salmonellosis, and is the parent strain of *Salmonella* Typhimurium SL1344 which is the WT strain which is used throughout this thesis (Jones, Collins and Aitken 1988, Rankin and Taylor 1966, Hoiseth and Stocker 1981). *S. enterica* serovar Typhimurium strains are the most commonly used strain for Typhoid fever research due to the fact that while it causes a gasteroenteric infection in humans it causes typhoid like symptoms in mice.

6.1.2 *Salmonella* Enteriditis

S. Enteritidis is responsible for approximately 14% of all *Salmonella* gasteroenteritis infections in the United States and is thought to be either the first or second most common *Salmonella* serovar in most countries (Braden 2006). Infection from *Salmonella* Enteritidis is highly associated with contamination from eggs, or more specifically, the eggshell.

6.1.3 *Salmonella* Dublin

S. Dublin is most commonly associated with infection in cattle but can also less commonly infect pigs and also humans. In cattle it causes an enteric fever and also commonly induces abortion (Coburn et al. 2007). Cattle frequently enter a chronic carrier stage, equivalent to that seen in humans post infection with *S.* Typhi, whereby they can shed the bacteria in large numbers and spread the infection quickly within a herd. The bacteria can also be passed through the milk and hence to calves or, if from dairy farms, to humans (Nielsen et al. 2004).

6.1.4 Salmonella Choleraesuis

S. Choleraesuis is a host adapted serovar which causes a systemic infection in swine. As well as the loss of life this disease causes, there is a large economic burden to the meat industry. In 1958 Choleraesuis was responsible for 90% of isolated causes of *Salmonella* in pigs, however by 1997 this had dropped to a single isolated case in the UK (Sojka et al. 1977).

6.1.5 Salmonella Gallinarum and Pullorum

Salmonella Gallinarum and Pullorum were originally thought to be two separate serovars but now are regarded as two biovars of the same serovar – that is they are indistinguishable by standard serotyping. However, they differ physiologically and biochemically (Barrow and Freitas Neto 2011).

S. Gallinarum causes typhoid in fowl, and can also rarely causes an infection in mice and humans. The disease is acute or chronic septicemia which generally affects adult birds. Interestingly the eradication of *S.* Gallinarum allowed for the increase of *S.* Enteritidis, and subsequent increase in human infections, as this strain could then fill the vacated ecological niche (Rabsch et al. 2000).

S. Pullorum is the causative agent of pullorum disease and contrary to Gallinarum causes a systemic disease more commonly seen in young birds.

6.1.6 *Citrobacter rodentium*

In addition to studying a number of *Salmonella* serovars we also look at *Citrobacter rodentium.* Since the *Salmonella* sytemic serovars Typhi and Paratyphi are Hazard Group 3 human pathogens these were inpractical for use in this study. *Citrobacter rodentium* provided us with a safe model of another systemic strain, as it a model for the emerging pathogen enterohemorrhagic *Escherichia coli* (EHEC) (Mundy et al. 2005). It is worth noting that previous work in the Rowley lab (Figure 3) has shown a signifciant difference between the amount of N_2O released by *Salmonella* and a K12 *E. coli* strain, what is not clear from that work is whether the reduced N_2O release from *E. coli* is indicative of the entire species or due to the fact that this is a commensal strain. This strain releases a toxin similar to the Shiga toxin produced by *Shigella dysenteriae* (O'Brien and LaVeck 1983). EHEC causes severe and bloody diarrhoea, haemorrhagic colitis (HC) and, haemorrhagic uraemic syndrome (HUS); the latter of which causes acute renal failure,thrombocytopenia, and microangiopathic haemolytic anaemia. The mortality from HUS is quite high at up to 17%, with a further 30% suffering from permanaent disabilites (Carter et al. 1987, Griffin and Tauxe 1991, Messens et al. 2015, Karmali 1989, Karmali, Gannon and Sargeant 2010, Welinder-Olsson and Kaijser 2005).

6.1.7 Differences in vitamin B_{12} synthesis between *Salmonella* serovars

S. Gallinarum and *S.* Pullorum are two host-restricted strains which cause a systemic infection in fowl. It has been noted that both serovars have developed pseudogenes which result in the loss of ability to synthesise Vitamin B_{12}, and hence this is a potential marker of a switch from an enteric to a systemic disease (Langridge et al. 2015). One of the functions vitamin B_{12} plays in *Salmonella* is in the degradation of 1,2-propandediol required for the use of tetrathionate as an electron acceptor. It is known that the ability to reduce tetrathionate was lost pre-divergence and the loss of B_{12} synthetic ability was lost at a later point (Langridge et al. 2015). We therefore believe it may be the case that systemic strains have lost the ability to synthesise vitamin B_{12} due to the fact that they have moved out of the intestine where vitamin B_{12} dependent tetrathionate respiration is of benefit (Nuccio and Bäumler 2014). This chapter aims to

build on this hypothesis and combine it with the possibility that the levels of N_2O produced by these strains may vary, due to its toxic effects on vitamin B_{12} (Page 36).

The vitamin B_{12} transporter, BtuB is present in EHEC and is a known virulence factor in this strain, highlighting an importance of acquisition of vitamin B_{12} from the external environment (Hochhut, Dobrindt and Hacker 2005), and there is evidence that EHEC cannot synthesise B_{12} de novo (Cordonnier et al. 2016). Vitamin B_{12} is important however for the induction of EutR which contributes to metabolism and virulence in EHEC (Luzader et al. 2013).

Nuccio and Bäumler's analysis revealed mutations in genes required for ethanolamine degradation, propanediol utilisation and vitamin B_{12} synthesis and regulation, in the systemic-disease causing strains: *S.* Choleraesuis, *S.* Gallinarum, *S.* Paratyphi A & C and *S.* Typhi, whereas the strains which cause an enteric infection, *S.* Agona, *S.* Enteritidis, *S.* Heideberg, *S.* Newport and *S.* Typhimurium, did not have mutations in these locations (Nuccio and Bäumler 2014). In this chapter we aim to measure the differing levels of vitamin B_{12} produced by different *Salmonella* serovars.

In Chapter 5 it was highlighted that since nitrate is present in the inflammed intestine, *Salmonella* will be able to use this to respire and in turn release high levels of nitrous oxide. As previously discussed it has been noted that *Salmonella* Typhimurium produces an order of magnitude more nitrous oxide then the close relative *E. coli*. We hypothesise that *Salmonella* produces this high level of nitrous oxide in order to compete with other gut bacteria by targetting the vitamin B_{12} pool. The question is therefore raised whether *Salmonella* serovars which cause systemic infections and therefore do not remain in the microxic environment of the inflammed intestine where nitrate is available, will produce similar levels of nitrous oxide.

6.1.8 Measurement of vitamin B_{12}

The most common reason for wanting to quantify the level of B_{12} in a sample is for medical purposes from a human blood. Here, two different measurements can be taken, total cobalamin in the blood, or cobalamin bound to the transport protein transcobalamin (termed holoTC). HoloTC is also referred to as active vitamin B_{12} as it is only in this form that it can be taken up by the human cells for use (Nexo and Hoffmann-Lücke 2011). The former is more common however the latter is more likely to give relevant information for medical purposes such as the diagnosis of Vitamin B_{12} deficiency (Nexo et al. 2002, Refsum et al. 2006).

High throughput medical labs use immunoassay based techniques for the quantitfication of Vitamin B_{12}, there are several commercially available kits. Examples include an electrochemiluminiscence immunoassay (ECLIA) measured on a Roche Elecsys 2010 (Roche Diagnostics, Basel, Switzerland), Vitamin B_{12} AccuBind ELISA Kits (Monobind, California, USA), Vitamin B_{12} ELISA (Eagle Biosciences, New Hampshire, USA). These assays are based on the instrinsic factor, which is the enzyme important for uptake of Vitamin B_{12} in humans. The main drawback of these assays for use in this study is price, with most costing many hundreds of pounds; it is also unclear whether these assays would be suitable for measuring the endogenously produced vitamin B_{12} by *Salmonella;* that is there is no mention of this in the literature.

Microbiological assays have also been used to measure vitamin B_{12}. One study used the protozoan *Ochromonas mahamensis* which requires B_{12} for growth and responds linearly to the vitamin B_{12} (FORD 1953). Mutant strains of *E. coli* have also been used (BURKHOLDER 1951). In general, these microbiological assays are not as accurate and take longer than immunoassays.

The measurement of vitamin B_{12} levels from bacterial cultures has not previously been performed in our lab group. This chapter includes the optimisation of a vitamin B_{12} quantification assay for measurements of vitamin B_{12} synthesised by *Salmonella*, based on a previously published protocol (Lawrence and Roth 1996). This assay is a disc diffusion assay based on the B_{12} dependency of a *Salmonella metE* mutant.

6.2 Aims

Building on the results from Chapter 5 this chapter aims to uncover if the hypothesis that *Salmonella* produces high levels of N_2O and vitamin B_{12} in order to outcompete the gut microbiota therefore means that systemic strains will not produce these compounds to the same extent. Specifically we will:

- Bioinformatically analyse the *cob* operon across multiple *Salmonella* serovars.
- Measure the levels of vitamin B_{12} and N_2O produced by a range of *Salmonella* serovars.

6.3 Results:

6.3.1 Systemic *Salmonella* serovars have mutations in the *cob* operon

An alignment of the *cob* operon between the 8 *Salmonella* strains studied in this chapter and also two further systemic strains, *Salmonella* Paratyphi A and *Salmonella* Typhi, was constructed to visualise differences between the operon in these strains (Figure 49). The percentage identities of each gene in comparison to SL1344 are shown in Table 10. In addition to the core *cob* operon (*cbiA – cobT),* two upstream genes, *pocR* and *pduF*, and one downstream, *erfK*, were included in the alignment.

RNA-seq analysis of the *cob* operon has revealed that a single transcript runs from the beginning of the operon and finishes after the *erfK* gene (Jay Hinton, personal communication), it is for this reason *erfK* was included in the alignment. In contrast, in *E. coli* it is most likely the transcript is halted before *erfK* (Lawrence and Roth 1995). There is very little in the literature about ErfK*,* except for the possible involvement of the protein in protection against bile (Hernández et al. 2012). The gene product of PocR is a positive transcriptional regulator of the *cob* operon, although is not essential for its transcription. PocR also regulates the *pdu* operon, responsible for vitamin B_{12} dependent propanediol utilisation (Rondon and Escalante-Semerena 1992, Bobik et al. 1999, Chen et al. 1995a, Escalante-Semerena and Roth 1987).

The entire *cob* operon was present in all the enteric strains, *S.* Typhimurium SL1344, *S.* Typhimurium 4/74, *S.* Dublin and, *S.* Enteritidis, with 98% or higher consensus for all genes. A complete *cob* operon is also present in the systemic swine strain, *S.* Choleraesuis, with 97% or higher consensus for all genes. The closely related strains *S.* Gallinarum and Pullorum both contain missing genes, the regulator *pocR* is absent in Gallinarum, and the first gene of the operon *cbiA* is absent in Pullorum. The two strains which cause systemic infections in humans, *S.* Typhi and Paratyphi both have numerous mutated genes; in *S.* Typhi *cbiB* and *cbiK*, and in *S.* Paratyphi *cbiA* and *pduF* (Figure 49*,* Table 10)*.* In all the cases of ‘missing’ or ‘mutated’ genes there are a large number of stop codons inserted into the DNA sequence, which has resulted in the gene being visible in some cases on the alignment (Figure 49), such as in the case of *S.* Typhi. However, it is very clear from viewing the DNA sequence that a functional mRNA would not be transcribed and therefore no protein would be produced.

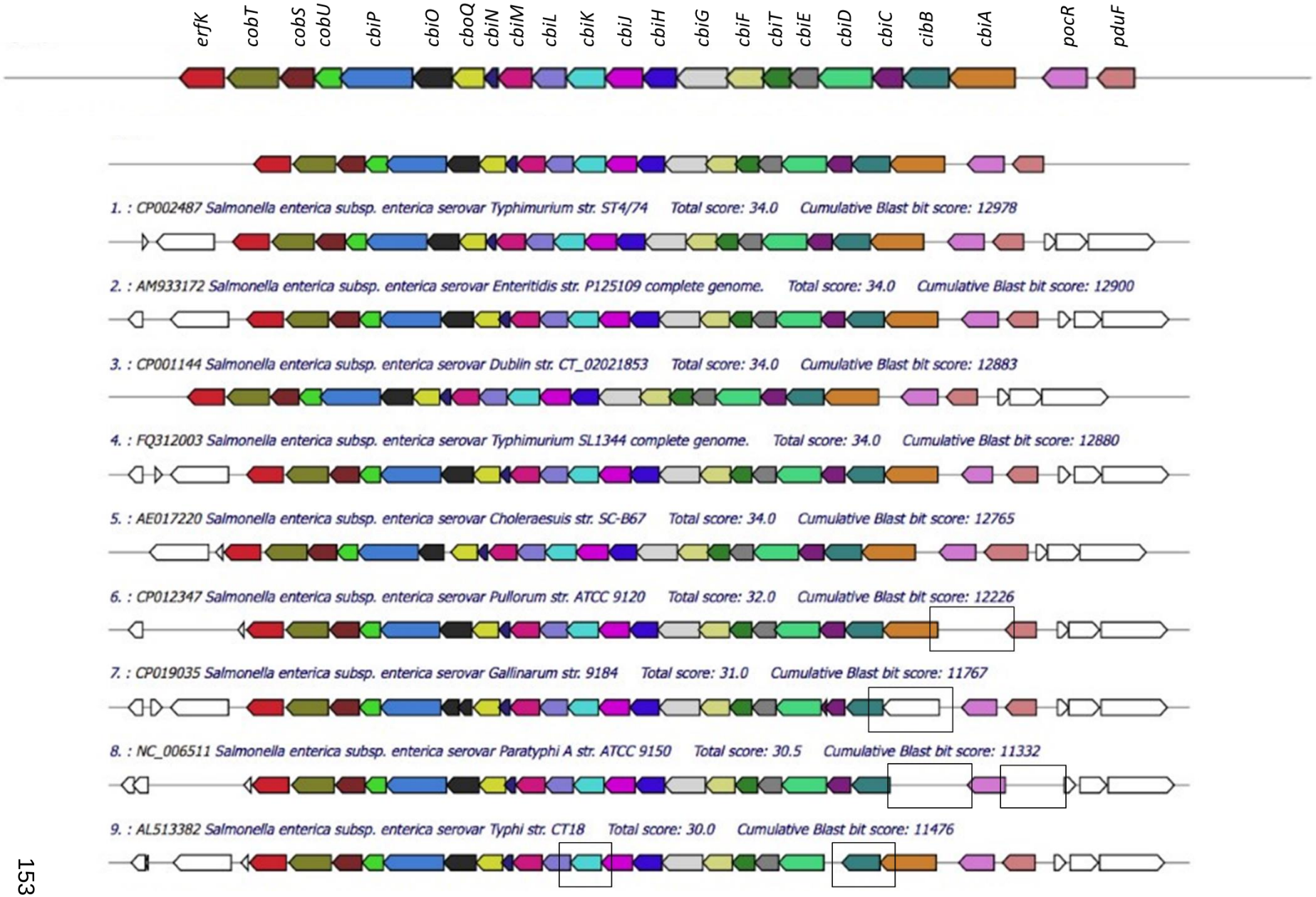


Figure 49 **Alignment of the *cob* operon between *Salmonella* serovars**. Gene names are shown by the top legend. All strains were compared to *Salmonella enterica subsp. enterica* serovara Typhimurium SL1344. Genes which are missed or highly mutated are highlighted by a black box. Constructed using MultiGeneBlast.

Table 10 **Genetic consensus of the *cob* operon across *Salmonella* serovars** Percentage identities (DNA) are shown. Genes with 0% identity are highlighted. Constructed via MultiGeneBlast.

	***S.* Typhimurium SL1344**	***S.* Typhimurium 4/74**	***S.* Enteritidis**	***S.* Dublin**	***S.* Choleraesuis**	***S.* Pullorum**	***S.* Gallinarum**	***S.* Paratyphi**	***S.* Typhi**
erfK	100	100	100	100	99	99	99	99	99
cobT	100	100	100	100	99	99	98	98	98
cobS	100	100	100	100	99	99	97	97	98
cobU	100	100	99	99	100	99	99	99	99
cbiP	100	100	98	98	99	98	99	99	99
cbiO	100	100	98	98	98	98	98	98	98
cboQ	100	100	98	98	98	98	99	99	99
cbiN	100	100	100	100	98	98	98	98	98
cbiM	100	100	100	100	99	100	100	100	92
cbiL	100	100	98	98	97	98	99	99	99
cbiK	100	100	99	98	100	98	98	98	0
cbiJ	100	100	99	99	98	99	98	98	92
cbiH	100	100	98	98	99	98	99	99	99
cbiG	100	100	99	98	99	98	99	99	99
cbiF	100	100	99	99	99	99	99	99	100

cbiT	100	100	100	100	100	99	100	100	99
cbiE	100	100	98	98	98	97	100	100	100
cbiD	100	99	99	99	99	99	98	98	98
cbiC	100	100	100	100	99	99	98	98	98
cibB	100	100	98	99	98	98	97	97	0
cbiA	100	100	99	98	98	98	0	0	99
pocR	100	100	100	100	99	0	99	99	99
pduF	100	100	100	100	100	99	99	0	99

6.3.2 Development and optimisation of a Vitamin B_{12} quantification assay

As discussed in the Introduction to this chapter, the measurement of levels of vitamin B_{12} produced by bacteria has not been done previously in our lab, and while there are commercially available kits these come at a high cost. We therefore worked to develop the protocol for a microbiological assay for B_{12} measurement which would be able to measure the B_{12} produced by *Salmonella*, based on a previously published protocol (Lawrence and Roth 1996). The assay is based on the vitamin B_{12} dependency of a *Salmonella* Typhimurium SL1344 *metE* mutant. This strain is spread on a minimal media plate which lacks vitamin B_{12} and a sample from the test strain placed on a filter disc. The growth of the *metE* strain can then be measured which correlates with the amount of vitamin B_{12} in the sample. The growth diameter of a range of standard vitamin B_{12} concentrations (A) and the resulting standard curve (B) are shown in Figure 50.

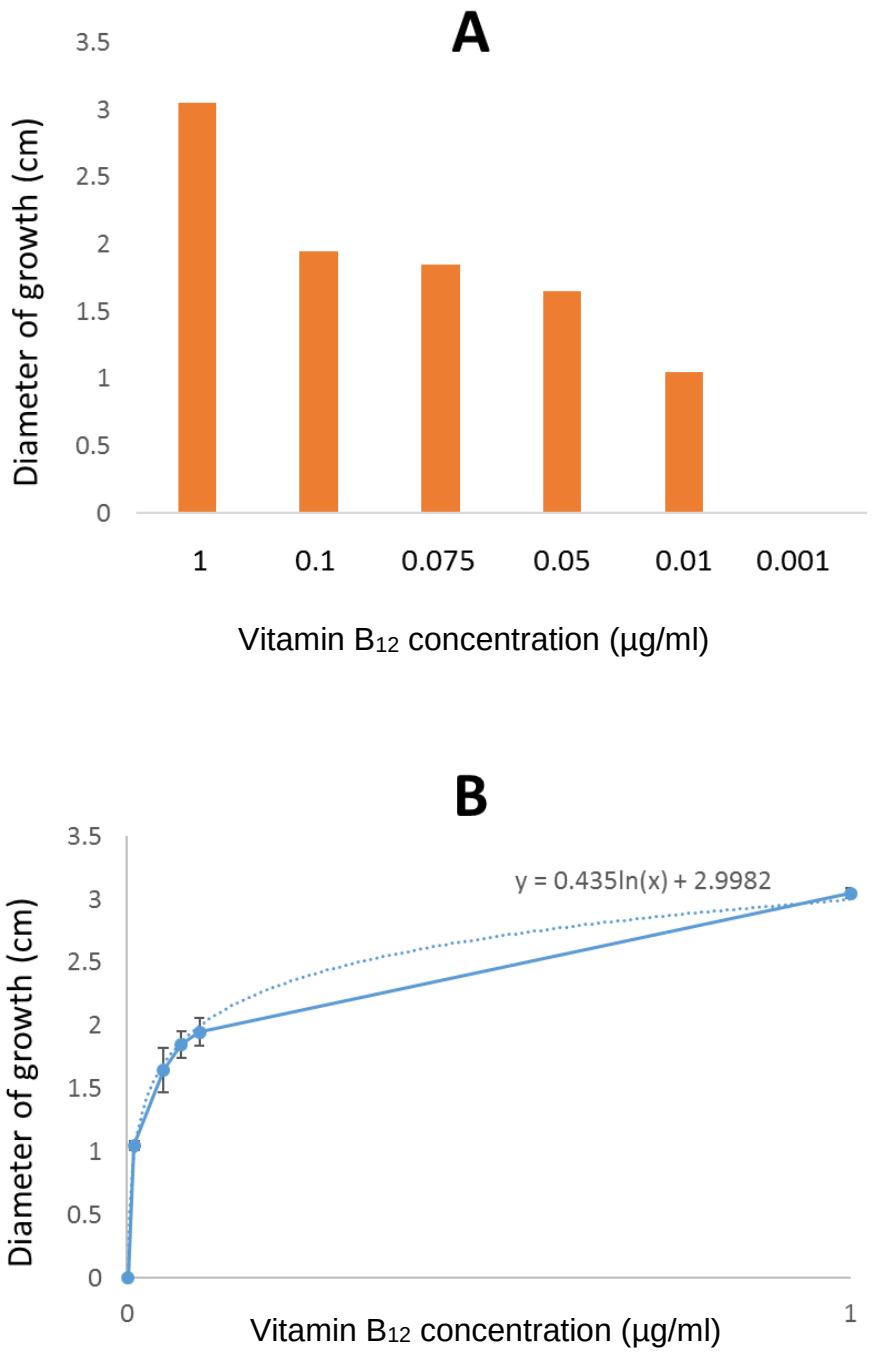


Figure 50 **Vitamin B_{12} bioassay.** A SL1344 Δ*metE* mutant was spread on an M9 minimal media plate. A disc is placed in the middle with a specific concentration of vitamin B_{12} and the resulting growth measured. Diameter of growth is shown in (A) and (B) for the construction of a calibration curve, error bars show SE.

6.3.4 Levels of Vitamin B_{12} produced by *Salmonella* serovars and *Citrobacter rodentium*

In order to comparatively measure the amount of vitamin B_{12} produced by the various strains, growth conditions had to be used under which all strains grew equally. There was no external methionine or vitamin B_{12} and, components for the synthesis of vitamin B_{12} were not limited (cobalt, 1,2-propanediol). M9 minimal medium was used, supplemented with an amino acid mix containing all 19 amino acids except methionine.

Under these conditions all strains grew comparably, that is there was no lag for any of the serovars which had been seen previously for *S.* Pullorum and *S.* Gallinarum in the absence of additional amino acids. All strains reached maximum OD by 24 hours so the assay was conducted at this timepoint.

While this assay cannot give a clear or accurate measure of the exact amount or rate of synthesis of vitamin B_{12} by each strain it is possible to compare relative levels between the strains and to calculate the concentration of vitamin B_{12} in the 10 µl sample using the calibration curve constructed (Figure 50).

The two *S.* Typhimurium strains, SL1344 and 4/74 produced the highest levels of $B_{12,}$ at 0.1 µg/ml and 0.14 µg/ml respectively. This was then followed by the four further 'enteric' strains, *S.* Dublin (0.070 µg/ml), *S.* Enteritidis (0.045 µg/ml) and *S.* Choleraesuis (0.025 µg/ml). Of the two 'systemic' strains, only *S.* Gallinarum showed any level of B_{12} production (0.013 µg/ml), no production was seen for *S.* Pullorum. *Cirtrobacter rodentium* produced the lowest measured level of B_{12} (0.011 µg/ml).

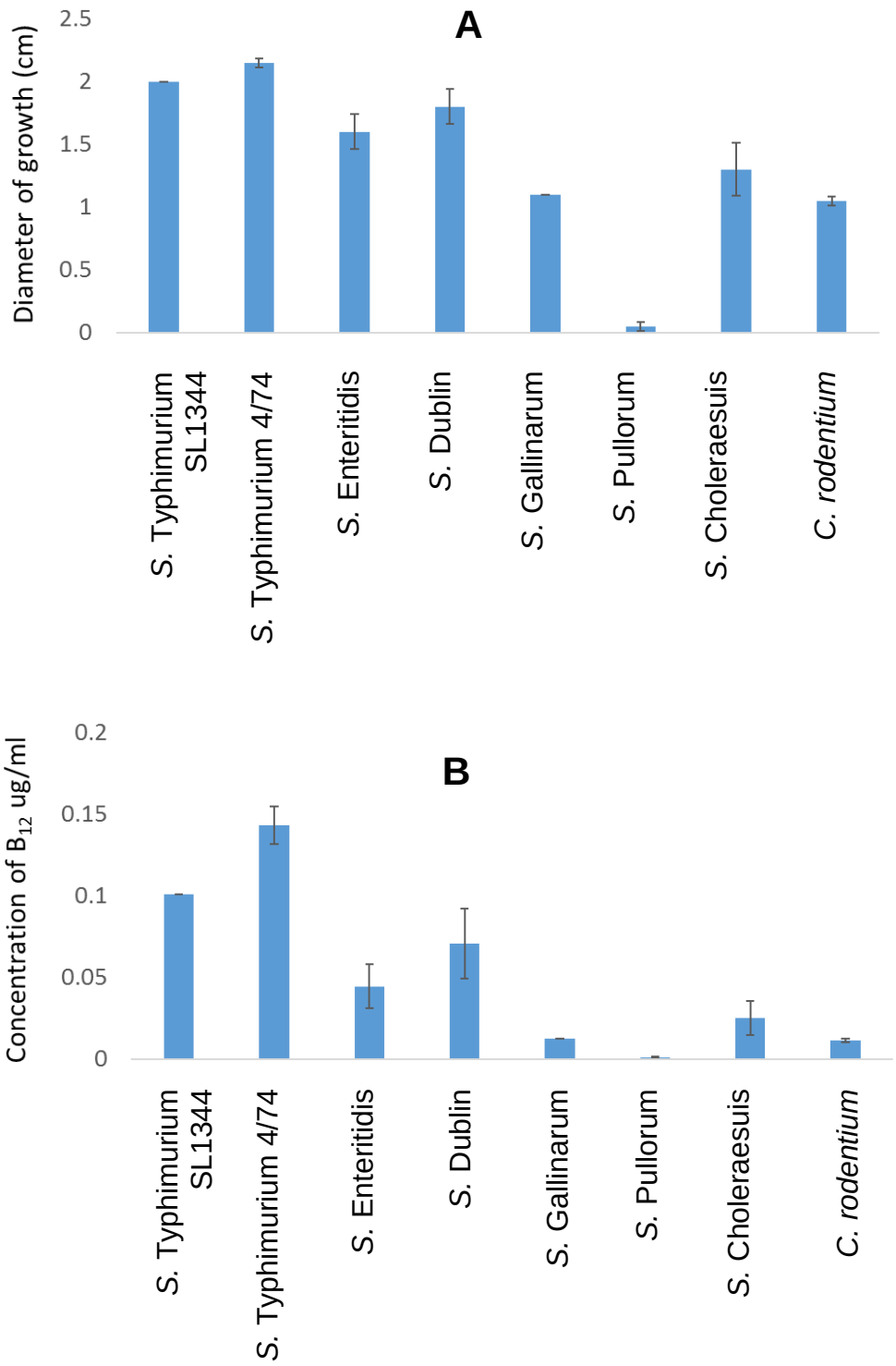


Figure 51 **Vitamin B_{12} bioassay.** A SL1344 Δ*metE* mutant was spread on an M9 minimal media plate. A disc is placed in the middle with a sample of lysed cells from the relevant strain. Protocol described in detail in Materials and Methods (Page 54). The diameter of the growth is shown in (A), and the concentration of the 10µl spot in (B), calculated using the calibration curve (Figure 50B). Data is representative of 3 repeats and SE is shown.

6.3.5 Comparison of NO and N_2O released by different *Salmonella* serovars during anaerobic nitrate growth

Salmonella serovars and *Citrobacter rodentium* were grown under denitrifying conditions to compare the levels of NO and N_2O released by each strain. Strains were grown in M9 minimal media supplemented with casamino acids to ensure comparable growth of all strains irrespective of amino acid requirements, 20 mM nitrate and 10 mM glycerol. We attempted to conduct this experiment without the addition of casamino acids to be more in line with other experiments conducted throughout this thesis however the growth rate of the different serovars was highly variable and therefore any attempt to draw comparisons would have been futile. With the addition of casamino acids growth was comparable between all strains (Figure 52).

Nitric oxide and nitrous oxide measurements were taken at 17, 24, 40 and 48 hours of growth, concentrations for each timepoint are shown as well as total NO/N_2O for the entire 48 hours (Figure 53 & Figure 54).

In general, a similar pattern is seen for the differences in NO and N_2O released by each strain, that is, the strains that release the most/least NO also release the most/least N_2O (Figure 53 & Figure 54). Additionally, all strains release greater levels of N_2O than NO, with an average difference of 7.7 fold. This pattern does not always hold true however; the WT strain used throughout this thesis, *S.* Typhimurium SL1344, produced the second highest level of N_2O but the least amount of NO, a 13 fold difference between the two gases. This suggests that there is, in comparison to the other strains, a higher rate of conversion of NO to N_2O. Surprisingly however, the opposite is seen for the parent strain *S.* Typhimurium 4/74 with the lowest levels of N_2O but the second highest level of NO, a 3.7 fold difference (Figure 53 & Figure 54).

Salmonella Pullorum exhibited the highest levels of both NO and N_2O release, totaling 108.7 µM and 1.0 mM respectively. The closely related strain, *Salmonella* Gallinarum which also causes systemic fowl infections, showed much lower levels, 67.3 µM NO and 0.47 mM N_2O. *Citrobacter rodentium* released NO and N_2O levels higher than all but 2 of the *Salmonella* serovars, 83.2 µM and 0.67 mM respectively (Figure 53 & Figure 54).

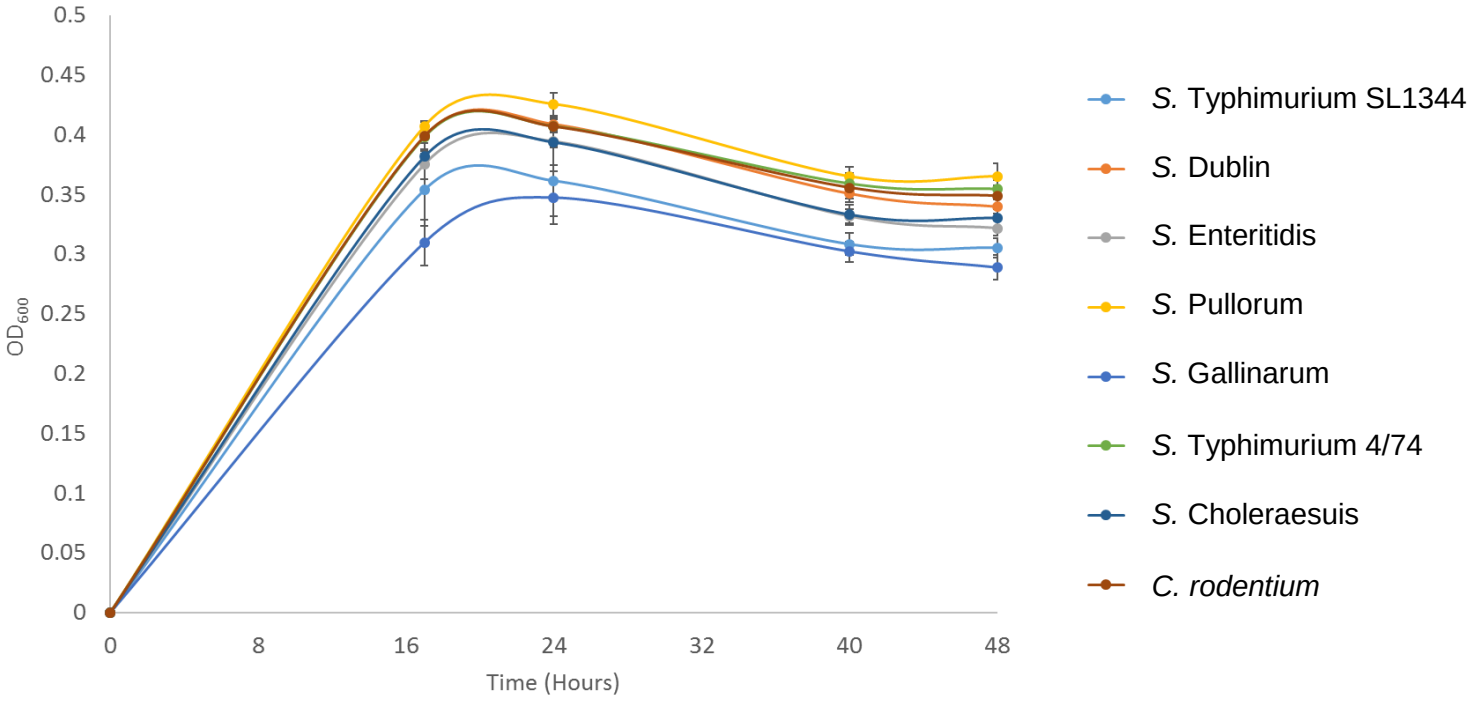


Figure 52 **All tested *Salmonella* serovars and *Citrobacter rodentium* grow comparably.** Strains were grown in nitrate-sufficient, glycerol-limited M9 minimal media supplemented with casamino acids. 3 repeats were used and SE is shown.

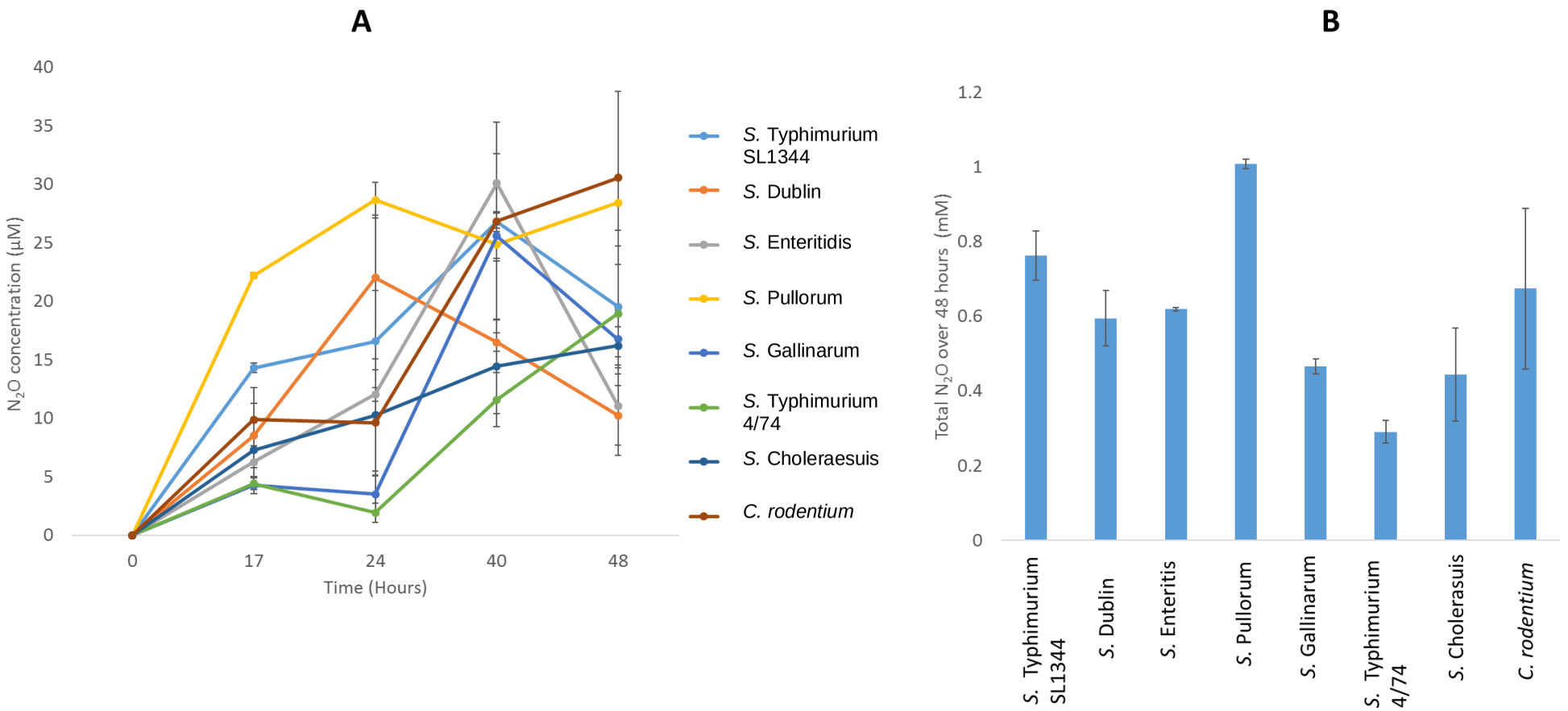


Figure 53 **Nitrous oxide levels of *Salmonella* serovars and *Citrobacter rodentium*. Strains** were grown in nitrate-sufficient, glycerol-limited M9 minimal media supplemented with casamino acids. 3 repeats were used and SE is shown. A) concentrations of N_2O over 48 hours, B) total N_2O released over 48 hours.

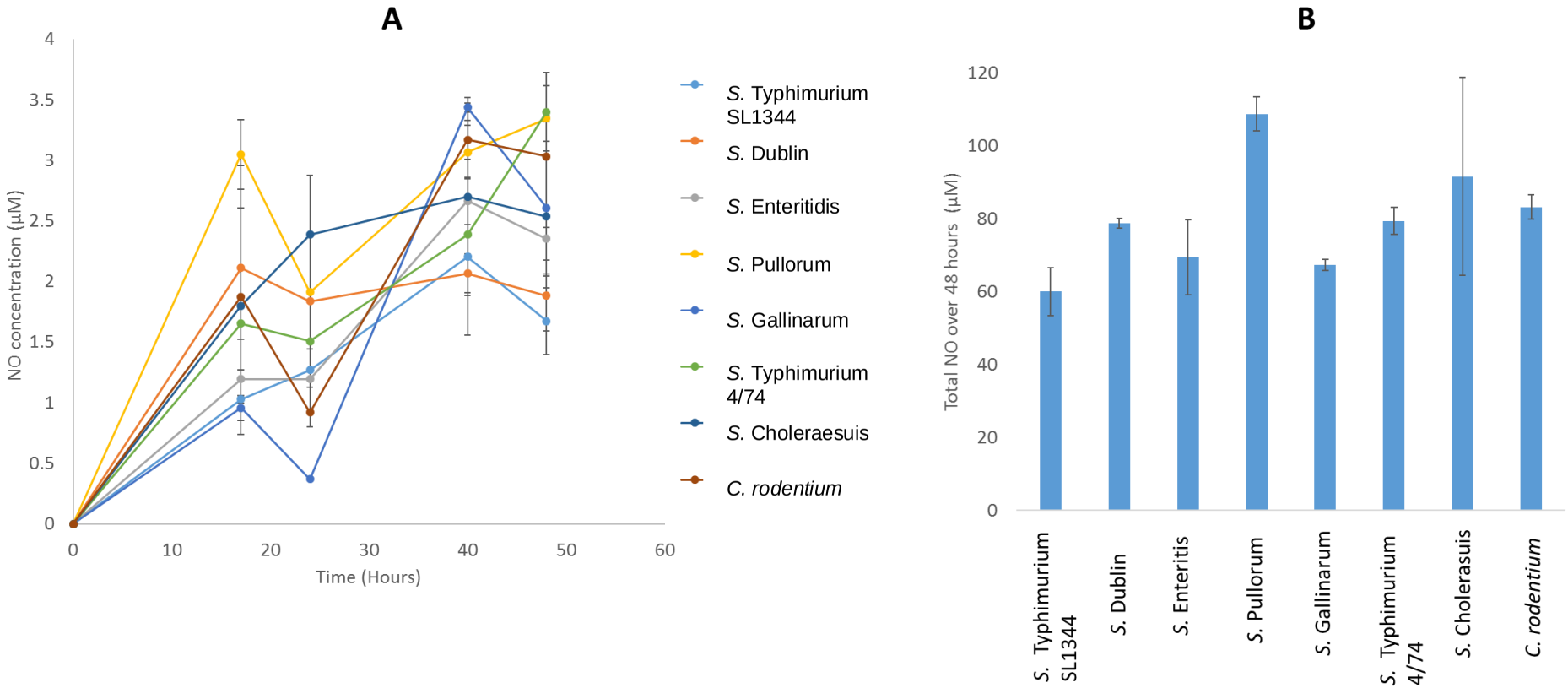


Figure 54 **Nitric oxide levels of *Salmonella* serovars and *Citrobacter rodentium*.** Strains were grown in nitrate-sufficient, glycerol-limited M9 minimal media supplemented with casamino acids. 3 repeats were used and SE is shown. A) concentrations of NO over 48 hours, B) total NO released over 48 hours.

6.4 Discussion

This chapter asked the whether there is a link between vitamin B_{12} production and the level of nitrous oxide released between different strains. This was partially based on the hypothesis raised in Chapter 5 that *Salmonella* produces this high level of nitrous oxide in order to compete with other gut bacteria by targetting the vitamin B_{12} pool. The potential is therefore raised whether systemic pathogens, including some *Salmonella* serovars which do not remain in the microxic environment of the inflammed intestine where nitrate is available, will produce similar levels of nitrous oxide to those that do not, and whether this is linked to their ability to produce vitamin B_{12}.

The vitamin B_{12} sythesis ability of different *Salmonella* serovars was discussed in the introduction of this chapter and built upon in the results. An alignment of the *cob* operon (Figure 49, Table 10) confirmed that pseudogenes have formed and genes been lost in 4 of the 5 systemic strains analysed, *Salmonella* Gallinarum, Pullorum, Typhi and Paratyphi, but not in *Salmonella* Choleraesuis. There are however a large number of mutations in the B_{12}-dependent systems required for propanediol utilisation and ethanolamine degradation in *S.* Choleraesuis (Nuccio and Bäumler 2014). It was interesting that especially in *Salmonella* Typhi the genes were not completely missing but instead have aquired large numbers of stop codons throughout the DNA sequence. All the mutated systemic strains had a mutation in either *cbiA* or *cbiB,* the first two genes of the operon, or in *pocR,* the positive regulator of the operon. A mutation at the beginning of the *cob* operon would result in the synthesis of the vitamin being halted in the very early stages, this would therefore stop the metabolically expensive process before it progressed very far. A further mutation further down the operon would have very little phenotypic effect at that point as the proteins work sequentially in respect to the order of genes in the operon.

The mutation of *pocR* in *Salmonella* Pullorum is interesting, this strain contained a complete *cob* operon except for this, and was not analysed in the Nuccio study. While it is known that *pocR* is a positive regulator of the *cob* operon it is not clear from the literature whether the translation of the operon is dependent on *pocR* or not (Rondon and Escalante-Semerena 1992, Chen et al. 1995a). This study assessed the importance of PocR for the synthesis of vitamin B_{12} in *S.* Typhimurium SL1344 and

concluded since the vitamin B_{12} addicted mutant *ΔmetE* when combined with a *ΔpocR* mutation was still able to grow in anaerobic minimal media conditons under which a *ΔmetE* Δ*cobS* mutant is not, that PocR is not an essential regulator of the operon and that there are other unknown regulators. In the case of *Salmonella* Pullorum it therefore may be the case that this mutation does not result in the total lack of ability to trancribe the *cob* operon and produce vitamin B_{12}, however we have not yet looked into what an alternative regulator might be and whether this holds true across multiple *Salmonella* serovars.

We then developed a bioassay which would enable us to compare vitamin B_{12} production between different *Salmonella* serovars (Figure 50) This assay, which is based on a previously described protocol (Lawrence and Roth 1996), uses the vitamin B_{12}-dependency of a *S.* Typhimurium *ΔmetE* strain in order to assay the presence of the vitamin in a sample. The main issue faced however was in growing the strains equally and ensuring conditions where the production of vitamin B_{12} was required and thereore should occur, and that all components of the synthesis (such a cobalt which is required as a cofactor) were not limited. The major drawback however of this assay is that it does no provide an exact amount of B_{12} produced by the strains or a production rate. This is due to the fact that cells from 200 ml of culture must be lysed to release the endogenously produced vitamin and this process involves boiling which may damage a percentage of the vitamin – all strains were treated equally however and so comparisions are warranted. From the final results of this (Figure 51) we can conclude a few things. Our WT strain, *S.* Typhimurium SL1344 and the parent strain, 4/74 produced the highest levels of B_{12}, this was followed by the other enteric strains, *S.* Dublin and Enteriitdis, and then *S.* Choleraesuis. *S*. Gallinarum produced a very low level of B_{12} and *S.* Pullorum showed no growth around the disc and so we conclude no measurable B_{12} was released. *Citrobacter rodentium,* a model strain for the systemic causing EHEC, produced the lowest measurable level of vitamin B_{12}. There is therefore a clear trend, that enteric strains which will spend long periods of time in the intestine, produce high levels of vitamin B_{12} and systemic strains, which will quickly disseminate into other parts of the body, produce much lower concentrations of B_{12}.

The next stage of this study was to compare the levels of nitrous oxide produced by the different strains while undergoing denitrification. The hypotheis was that since we believe *Salmonella* Typhimurium releases such high levels of N_2O in order to compete

with the gut microbiota by targetting the vitamin B_{12} pool of these B_{12}-auxotrophs, that non-enteric strains which do not remain in the intestine will not benefit from this survival advantage of high N_2O emmisons and hence will release lower N_2O.

In general this hypothesis held true with the systemic strains producing less total N_2O than the enteric strains (Figure 53), however not in all cases. Our WT strain *S.* Typhimurium SL1344, produced the second highest level of N_2O whereas the parent strain *S.* Typhimurium 4/74 released the lowest levels of N_2O. The latter of this is quite surprising and further study should be done to confirm the result. The two further enteric strains, *S.* Dublin and *S.* Enteritidis then produced the next highest levels of N_2O, distinctly higher than *S.* Choleraesuis and *S.* Gallinarum, two systemic strains. However, two systemic strains, *S.* Pullorum and *Citrobacter rodentium* released two of the highest levels of N_2O. This study needs to be repeated to draw any strong conclusions, steady state culturing may also provide more useful insight into any differences between the strains.

Taken in combination the results of this chapter potentially provide insight into a link between vitamin B_{12} production and N_2O release throughout systemic and enteric strains. It is clear from previous studies, our alignment and the bioassay conducted that enteric strains are capable of producing B_{12} and at higher levels than the systemic strains. We cannot conclude from this data whether the reason for this phenomenon is correlated to a change in the production of N_2O by the strains or not. However, it seems clear that since the systemic strains have lost the ability to synthesise vitamin B_{12} this must not be of importance to their survival and pathogenesis.

Assessing the survival of the different strains when challenged with N_2O could reveal further insights in to their interactions with this gas, and moreover competition studies with commensal gut bacteria would be of interest. Do the enteric strains outcompete the gut microbiota, whereas the systemic strains do not – and would this difference disappear in the absence of available nitrate?

A further question arises whether this link between N_2O production and B_{12} synthesis is present throughout a wider range of pathogenic bacteria, particularly enteric pathogens. Most *Campylobacter* species are disease-causing, including *C. jejuni*, one of the primary causes of foodborne disease in humans and *C. coli* which also causes

an enteric infection in humans (Skirrow 1977). *Campylobacter* species are unable to produce both N_2O and vitamin B_{12} (Payne et al. 1982, Rodionov et al. 2003).

The enteric pathogen *Clostridium difficile* causes similar symptoms including watery diarrhea, fever and abdominal pain; it makes up about 20% of cases of antibiotic-associated diarrhea (Kelly, Pothoulakis and LaMont 1994, Vogel 1995). *C. difficile* can synthesise vitamin B_{12}, however analysis of its genome shows that both NarG and NapA are absent and it is therefore unlikely this strain can produce N_2O (Rodionov et al. 2003)

The enteric pathogen *Vibrio cholera* is the causative agent of cholera, which causes vomiting and diarrhea, and has a fatality ratio of approximately 4% (WHO). *V.* cholera can produce B_{12} (Rodionov et al. 2003) but only contains the periplasmic nitrate reductase (Nap) enzyme (Braun and Thöny-Meyer 2005) and so, it could be expected to produce lower levels of N_2O. *V. cholera* can however produce NO and is able to reduce N_2O and use it as an electron acceptor (Janoff et al. 1997, Kaspar and Tiedje 1981, Yoshinari 1980).

From the three common enteric pathogens mentioned here it would appear that this phenomenon does not translate to other strains. Another member of the Enterobacteriaceae family, to which *Salmonella* and *E. coli* belong, *Klebsiella pneumonia,* does produce both N_2O and B_{12}. When undergoing denitrification *Klebsiella* converts ≈5% of the nitrate to N_2O, a similar level to measured for *Salmonella* in this study (Satoh, Hom and Shanmugam 1983). *K. pneumonia* is found in our normal microbiota, in the mouth, skin and gut; however if inhaled it can result in pneumonia which carries a fatality rate of abput 50% (Meatherall et al. 2009). *Klebsiella* can also cause urinary tract infections, particularly in diabetics, and can lead to bladder or kidney stones (Ronald 2003). This strain is therefore not an enteric pathogen but contains the characteristics we see from enteric *Salmonella* strains.

There are many non-enteric, or systemic, pathogens which are unable to produce B_{12} including pathogenic *Neisseria, Hemophilus influenza* and *Helicobacter pylori* and *Bordetella pertussis*. The latter three of these are unable to produce N_2O, *Neisseria* can reduce NO to N_2O but cannot reduce nitrate, potentially resulting in low N_2O emissions (Rodionov et al. 2003, Stewart 2003, Barth, Isabella and Clark 2009, Marais et al. 1999). In contrast, the non-enteric bacteria *Pseudomonas aeruginosa,* which

causes numerous infections in the urinary tract, skin wounds, blood and, airways, particularly of cystic fibrosis patients, produces both B_{12} and N_2O (Bodey et al. 1983, Zumft 1997).

It therefore seems that while in *Salmonella* there is a link between B_{12} and N_2O production and being the causative agent of an enteric infection, throughout all bacteria this may be a more general link to pathogenicity.

In *Salmonella,* vitamin B_{12} is required for the degradation of 1,2-propandediol required for the use of tetrathionate as an electron acceptor, which is present in the inflamed intestine. The loss of ability to reduce tetrathionate came at an earlier stage than the loss of B_{12} synthetic ability (Langridge et al. 2015). With the conclusions drawn from this chapter we therefore believe it may be the case that systemic strains have lost the ability to synthesise vitamin B_{12} due to the fact that they have moved out of the intestine where as well as vitamin B_{12} dependent tetrathionate respiration providing a growth advantage, N_2O is released targeting any exogenous B_{12} (Nuccio and Bäumler 2014). If this is the case it could then be that enteric *Salmonella* strains which already require B_{12} provide themselves with a pathogenic advantage by producing high levels of N_2O – something that provides no advantage, but a clear detriment to non-B_{12} producing, systemic strains.

7 General Discussion

As each chapter has already been individually discussed this general discussion will summarise the major results of the study, contextualise the results in the wider body of *Salmonella* research, and provide suggestions for future studies.

7.1 Context

Salmonella is a global pathogen which causes a range of diseases in humans and animals. In humans, there are two major disease types, gastroenteritis and an systemic infection known as Typhoid fever. Enteric infections are generally self-limiting, and apart from infections of the immunocompromised, elderly or young, do not require any treatment. Typhoid fever however is a much more serious infection which without treatment usually results in death, and as such there are ≈200,000 global deaths annually.

The development of antibiotic resistant strains is an increasingly severe problem across a large number of pathogenic bacteria, primarily due to increased use and misuse of antibiotics.

There are also multi-drug resistant strains of *Salmonella* which are resistant to the so called ‘last resort’ antibiotics and therefore are untreatable. Current vaccines against Typhoid fever have numerous drawbacks, including not providing life-long resistance and hence being of limited use to those living in the endemic regions.

It is therefore of key global interest to better our understanding of how *Salmonella* causes infection in order to develop new treatments, including, but not limited to antibiotics, and vaccines.

7.2 Nitric oxide

Nitric oxide (NO) is a free radical which has both cytostatic and cytotoxic activities and causes widespread damage to numerous cellular targets including DNA and proteins (Fang 2004). *Salmonella* is exposed to NO both endogenously and exogenously throughout its lifecycle. As a facultative anaerobe, *Salmonella* has the ability to use alternative electron acceptors for respiration when oxygen is unavailable, one of these is nitrate. The respiration of nitrate in *Salmonella*, referred to as truncated denitrification, involves the sequential reduction of nitrate via nitrite and nitric oxide to

nitrous oxide. Hence the endogenous reduction of NO is required whenever *Salmonella* is respiring using nitrate. Additionally, the release of NO is an essential part of the host immune system particularly by macrophages. *Salmonella* have the ability to reside, survive and replicate within host macrophages, and rely on this ability in order to cause a systemic disease. NO release by macrophages is via the inducible NO synthase, iNOS. iNOS has been shown to be of particular importance to the host immune defence system in mice, with mice deficient in iNOS being significantly more susceptible to *Salmonella* infection (Alam et al. 2002, Mastroeni et al. 2000).

There are three well characterised enzymes for NO detoxification in *Salmonella*, HmpA, NorV and NrfA, the triple deletion of which does not render *Salmonella* completely unable to withstand NO stress (Mills et al. 2008). This therefore suggests there are further systems at play to enable *Salmonella* to detoxify NO.

Since NO detoxification is an essential step during *Salmonella* pathogenesis a better understanding of this process could allow the development of antibacterial drugs or vaccines that can target the enzymes involved in NO defense.

7.3 Nitrous oxide

Nitrous oxide is produced by *Salmonella* by the reduction of nitric oxide, primarily carried out by Hmp and NorVW. In *Salmonella*, due to the lack of a nitrous oxide reductase, denitrification is truncated and hence nitrous oxide is the end product. Similarly to *Salmonella, E. coli* also lacks a nitrous oxide reductase and hence has a net release of N_2O; however a combination of studies by (Rowley et al. 2012) (Stremińska et al. 2012) and Runkel et al (personal communication) has identified that nitrous oxide production by *Salmonella* Typhimurium cultured in nitrate sufficient/glycerol limited chemostats is an order of magnitude higher than that produced by related non-pathogenic *E. coli* strains (Figure 3).

In fact it has been observed that *Salmonella* also produces high levels of NO, and has been hypothesised that this is in order to enable the bacteria to produce such high levels of nitrous oxide and if so, for what purpose (Maia and Moura 2014).

7.4 Vitamin B_{12}

Salmonella is one of a reasonably small number of bacteria that is able to synthesise vitamin B_{12} *de novo.* B_{12} synthesis is a highly metabolically expensive process, the vitamin itself is very large, with a molecular weight of 1355.37 g/mol, and the process involves 30 different genes, which together make up over 1% of the *Salmonella* genome. Curiously however the requirements for vitamin B_{12} in *Salmonella* are very low. There are only four B_{12} dependent processes - vitamin B_{12}-dependent methionine synthase, MetH, ethanolamine ammonia lyase, which degrades ethanolamine (Roof and Roth 1989), propanediol dehydratase which allows *Salmonella* to use propanediol as a carbon source, and queuosine synthase, which produces queuosine, a nucleoside found in four tRNAs which is not essential for growth under laboratory conditions (Frey et al. 1988).

It was not until 2001 that a deleterious effect of losing any of these processes was first noted and therefore the beginning of an explanation as to why *Salmonella* produces vitamin B_{12}. Tetrathionate is an alternative electron acceptor which is produced in the inflamed intestine, the ability of *Salmonella* to respire using tetrathionate is dependent on either ethanolamine or propanediol the utilisation of which are both vitamin B_{12} dependent processes (Price-Carter et al. 2001, Thiennimitr et al. 2011).

However, the ability to respire on tetrathionate alone does not seem sufficient to warrant the synthesis of vitamin B_{12}. An observation was made in *Paracoccus denitrificans* that N_2O accumulation induces a switch from vitamin B_{12}-dependent to vitamin B_{12}-independent biosynthetic methionine pathways (Sullivan et al. 2013); this therefore suggested a possibility for a similar phenomenon in *Salmonella*, and a link between nitrous oxide, vitamin B_{12} and methionine requirements.

7.5 Characterisation of NO detoxification enzymes

The basis of this work was a prior study done in the Rowley group where a microarray was conducted in order to identify novel genes involved in the anaerobic protection against nitric oxide. Six genes highlighted from this analysis were further investigated during this study, *cstA, hcr, STM1273, yeaR, tehB* and *STM1808,* the latter three of which have the same annotated function as tellurite resistance genes.

This study created knockout mutants of each of these six genes and a *ΔtehB ΔSTM1808 ΔyeaR* triple mutant strain. Aerobically no growth defect was observed when *Salmonella* was challenged with NO in the absence of these three genes, even in combination. However, anaerobically, there is a clear defect for a triple *ΔtehB ΔSTM1808 ΔyeaR* mutant growing in the presence of 10 mM of the NO donor deta NONOate. Since there is no phenotype visible for the single mutants we conclude that there is functional overlap between TehB, STM1808 and YeaR in anaerobic nitric oxide protection. Further experiments then measured the amount of NO released by the different strains when growing under denitrifying conditions and once more the triple mutant strain showed significantly higher levels of release than the WT.

Three further genes which were upregulated by either exogenous or endogenous NO were then investigated. We confirmed a mild role for all three, *cstA, hcr* and *STM1273,* in both aerobic and anaerobic NO protection. The knockouts were only assessed as single mutants, as opposed to in combination with either other well-known NO detoxification systems or those identified in this study. It could therefore be the case that more pronounced phenotypes would become visible when combining some of these mutations, as seen for the Te^R strains.

The clearest role for any of these three proteins in direct NO reduction was for Hcr. When grown under denitrifying conditions the *Δhcr* mutant releases significantly higher levels of NO than the WT strain, thus suggesting that Hcr directly detoxifies NO and hence this strain has an impaired ability to do so. It is interesting that both the CstA and STM1273 knockout strains in fact release slightly lower levels of NO than the WT, indicating that these two proteins do not directly reduce endogenous NO.

STM1273 was only upregulated in the microarray conducted after exogenous NO exposure, not during endogenous exposure during denitrification. If STM1273 is present in the periplasm as opposed to cytoplasmic membrane this would further strengthen this hypothesis, and the cellular localization of this protein needs to be determined.

It is worth considering how the various NO protection systems across all bacteria function. The most common proteins are globins, heme-containing globular proteins which are important for transporting oxygen. The most well characterised and often said to be key NO reduction enzyme in *Salmonella* is Hmp, a flavohaemoglobin.

Additional globin proteins with roles in NO tolerance include HbN from *Mycobacterium bovis* and Vgb from *Vitreoscilla* sp (Ouellet et al. 2002) which similarly to Hmp convert NO into nitrate (Frey et al. 2002). However, additionally there are globin proteins which function in other ways such as Myoglobin and haemoglobin in animals which is thought to have a role in NO scavenging (Brunori 2001) and, Cgb in *Camplylobacter jejuni,* which confers resistance through an unknown mechanism (Elvers et al. 2004). A second group of NO protection enzymes are the reductases, this include NorV and NrfA from *Salmonella* and are capable of directly reducing the compound either to nitrous oxide or ammonia.

The next step is to determine the exact mechanism of how the six proteins included in this study provide *Salmonella* with protection against NO. Protein film voltammetry can be used in order to determine and quantify the NO reductase activity, as has previously been done for NrfA from *E. coli* (van Wonderen et al. 2008). This technique works by immobilizing the enzyme onto an electrode, placing this in the potential substrate (NO) and monitoring any charge produced due to the flow of electrons and thus the redox potential (Angove et al. 2002). In addition, the effect of changing conditions such as pH or other inhibitors can be assessed. Understanding the mechanism by which these proteins provide protection against NO is important for building a complete picture of NO resistance in *Salmonella* – especially if these different systems are acting in different ways at different parts of the cell. It would be the case if designing vaccines or drug targets based on any of these systems that one must be aware of how the numerous proteins interact, especially if they are able to compensate for the loss of others.

7.6 A link between nitric oxide and tellurite resistance in *Salmonella*

Three of the proteins shown in this study to be involved in NO protection are annotated as tellurite resistance proteins. We therefore also assessed the impact a loss of these proteins has on *Salmonella* growing in the presence of potassium tellurite. The results of this study show a clear link between tellurite and nitric oxide resistance in *Salmonella*.

We initially became interested in tellurite due to the fact that three of the proteins upregulated in response to NO, and discussed above, are annotated as tellurite

resistance genes. Tellurite is highly toxic to a wide range of bacteria; the causation of the toxicity is not currently clear but there are multiple resistance systems across the bacterial kingdom. The results presented here confirm TehB, STM1808 and YeaR to be important for tellurite protection in *Salmonella*, both aerobically and anaerobically. Previous work has suggested that the version of TehB in *Salmonella,* as opposed to the *E. coli* homologue*,* may not have the ability to protect against tellurite due to differences in the SAM-methyltransferase domain (Karlinsey et al. 2012). Conversely, we found TehB to afford *Salmonella* with the highest level of protection against tellurite.

We then hypothesised that since tellurite resistance proteins also afford *Salmonella* an advantage in the protection against NO that the reverse may also be true, that proteins known to play a role in NO protection may also provide tellurite resistance. The most striking result here was that of Δ*nsrR.* NsrR is the master negative regulator of NO detoxification in *Salmonella* and is responsible for regulating *hcp-hcr, ytfE*, *ygbA, hmp*, *yeaR-yoaG and STM1808* (Karlinsey et al. 2012). When challenged with tellurite Δ*nsrR* grows considerably better than WT. This therefore shows that the upregulation of these genes results in *Salmonella* being able to survive in the presence of this toxin. This was further investigated using numerous knockout strains which showed that YtfE, NrfA and NorV all exhibit a small but measureable degree of protection against tellurite anaerobically. The three proteins whose role in NO protection was confirmed in this study, CstA, Hcr and STM1273, also were shown to provide tellurite resistance.

7.7 Nitrous oxide

The second aspect of this thesis was looking at the role of nitrous oxide production in *Salmonella*. Previous studies have highlighted that *Salmonella* produces and releases nitrous oxide at high levels – but have been unable to provide an explanation as to why this might be. From the results presented here we hypothesise that when residing in the inflamed intestine, *Salmonella* releases N_2O in order to outcompete the resident gut microbiota.

The key protein linking nitrous oxide, vitamin B_{12} and the intestine together is MetE. Since nitrous oxide binds to and inactivates vitamin B_{12}, MetH, the vitamin B_{12}

dependent methionine synthase is unable to function adequately in its presence, however since *Salmonella* possesses a vitamin B_{12} independent methionine synthase, MetE, this protein is able to perform this vital function. A key finding of this work was the importance of MetE for denitrifying *Salmonella*, where a clear growth defect for *ΔmetE* was seen, a defect which was further amplified by saturating the growth media with N_2O.

The regulation of vitamin B_{12} synthesis was also investigated. Previous studies have highlighted PocR as being the primary regulator of the *cob* operon and hence of vitamin B_{12} synthesis. However, results from this study suggest that there are alternative regulators due to the fact that the absence of PocR is not detrimental for the vitamin B_{12} addicted strain, *ΔmetE,* and in fact provides a slight restoration of the growth defect. We provide a potential explanation that when PocR is present it binds in such a location that it blocks the binding sites for other unknown regulatory elements. The full understanding of the regulation of the *cob* operon may then provide insights into when and why this operon is active.

Based on the hypothesis drawn we then queried whether the production of vitamin B_{12} and N_2O is similar throughout a range of *Salmonella* serovars, in particular if systemic strains produce these two compounds at the same rate as enteric strains. Systemic strains of *Salmonella*, such as *Salmonella* Typhi the causative agent of Typhoid fever, do not remain in the inflamed intestine where nitrate is available for extended periods of time, and instead reside in areas of the body where oxygen is likely to be more abundant.

By looking both bioinformatically and by the development of a vitamin B_{12} bioassay we concluded that there is a general trend for enteric *Salmonella* serovars to produce higher levels of vitamin B_{12} than the systemic strains. Both *S.* Typhi and *S.* Paratyphi could not be assessed in our hands but bioinformatic analysis would suggest that they are unable to produce any vitamin B_{12}. There was a less strong correlation for the amount of N_2O produced by the different strains although a general trend could be seen with the enteric strains releasing higher levels of N_2O than the WT strain. What is of most interest here is that the systemic strains must not require vitamin B_{12} and hence have acquired pseudogenes or lost genes in the *cob* operon, this further indicates the important of vitamin B_{12} for *Salmonella* residing in the intestine.

Initial future experiments should be based on competing *Salmonella* against members of the gut microbiota under denitrifying conditions as would be found in the inflamed intestine. Bioinformatic analysis of common gut microbiota revealed that almost no members of the microbiota have the vitamin B_{12} independent enzyme, MetE (Table 9). In addition, none of the microbiota we analysed possess NosZ, the enzyme required for the reduction of nitrous oxide. It therefore is highly likely that when growing on nitrate *Salmonella* will be able to outcompete the gut microbiota. Since it would be very complex to assess this interaction *in vivo* an *in vitro* approach would be required. A simple co-culture experiment could be used, whereby the same experimental setup used in this study is used but with commensal gut bacteria and *Salmonella* cultured in the same vessel. This would require thorough experimental design to ensure conditions which allowed for both bacteria to grow, that is that when cultured separately the commensal bacteria exhibits normal growth. One drawback of this set up is that it does not closely mimic the natural conditions of the gut, where a complex community of gut commensal bacteria are situated with a small number of *Salmonella* infecting. A preferential system would be a model where a defined microbiota could be allowed to become established and then a later inoculation of *Salmonella* be made, and the effect of the pathogen on the microbiota numbers monitored. One system which could allow for this would be a microfluidics gut model, where media is constantly being pumped through the system and thus bacteria can survive for extended periods of time. This is a new and rapidly developing field but a number of groups are having successes with similar studies (Shah et al. 2016, Kim and Ingber 2013, Kim et al. 2016a).

Based on the general hypothesis drawn and also the significant reduction in survival seen for a *ΔmetE* mutant in macrophages it would also be of interest to monitor this strain in the mouse model. In particular this could be used not just to monitor the survival of the mice, but to track the ability of the bacteria to disseminate from the intestine. Analysis of the mouse stools to measure bacterial diversity and quantity could also be used to give some information on how this strain, in comparison to the WT, impacts on the mouse microbiota.

There are three potential ways to try and target *Salmonella* using this information. One, by increasing the levels of vitamin B_{12} available, two, by removing the N_2O from the

intestine or three, by providing the commensal bacteria with a way by to survive even with high levels of N_2O and low levels of vitamin B_{12}.

Since we have shown that *in vitro* the effect of N_2O can be overcome with the addition of vitamin B_{12} there is the potential that this could be the case in the intestine. Vitamin B_{12} could be delivered to the intestine in high levels, either orally in a prebiotic fashion, or in combination with antibiotics, and this may be able to negate the advantage *Salmonella* has over the non-vitamin B_{12} producing commensal bacteria.

Another possibility is reducing the amount of N_2O present. There is a wide range of work looking at the production and release of N_2O into the atmosphere from agricultural soils due to its function as a greenhouse gas; and therefore, research into possible mitigation strategies (Ravishankara et al. 2009). The most feasible strategies are those which rely on the natural bacteria N_2O reductase system, NosZ, and increasing the expression and function of this enzyme. It may be possible to engineer a member of the gut microbiota to express *nosZ*. This would allow the gut bacteria to reduce N_2O to N_2. This could then be used as a probiotic strain which would be able to remove the N_2O which *Salmonella* emits from the intestine and negate the impact this has on the wider vitamin B_{12} pool.

Additionally, providing important members of the gut microbiota with MetE, the vitamin B_{12} independent methionine synthase would allow them to survive even in the presence of N_2O. The creation of genetically modified, so called ‘smart’ probiotic strains is controversial and not yet well studied; any work in this field would need to do thorough testing to ensure that the modification to the bacteria does not result in a conversion to a pathogenic phenotype, nor that the gene introduced cannot be transferred to other bacteria. There are however increasing numbers of studies looking to modify probiotic bacterial strains (Kullen and Klaenhammer 2000, Steidler and Rottiers 2006, Durrer, Allen and Hunt von Herbing 2017, Hwang et al. 2017)

7.8 Survival within macrophages

Numerous strains constructed throughout this study were tested for their ability to survive within murine macrophages. The ability for *Salmonella* to survive within the host environment of the macrophage is essential to its virulence (Fields et al. 1986) and therefore this is a key feature in the pathogenicity of the pathogen and potential

source of a vaccine stain. The most severely attenuated strain is the *ΔtehB ΔSTM1808 ΔyeaR* triple tellurite mutant with a four-fold reduction in bacterial survival compared to the WT strain. Approximately a two-fold reduction was seen both for *Δhcr* and for *ΔmetE* when methionine was lacking from the cell culture medium. These phenotypes are all of worth pursuing to assess if the phenotypes translate to a reduced survival of these strains in a mouse model. The combination of some of these mutations, or the addition of knockout mutation of proteins such as HmpA may also further amplify the phenotypes seen.

7.9 Final conclusions

In conclusion, this study highlights two aspects of denitrification in *Salmonella* which may contribute to pathogenesis. We have characterised novel nitric oxide detoxification proteins and shown a clear link between nitric oxide and tellurite protection in *Salmonella*. We have also provided an explanation for two phenomena, the production of vitamin B_{12} and the production of high levels of N_2O. We hypothesise that these two compounds afford *Salmonella* an advantage when residing in the inflamed intestine.

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