A correlation between tellurite resistance and nitric oxide detoxification in *Salmonella* Typhimurium

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Abstract

Salmonella are important enteric pathogens that are responsible for causing various diseases from gastroenteritis to systemic typhoid fever. *Salmonella* are a major contributor to morbidity and mortality worldwide. Crucial to their pathogenesis is the survival in harmful conditions elicited by the host immune system, one of these being reactive oxygen and nitrogen species (ROS/RNS). These are produced by macrophages and neutrophils in an attempt to eliminate pathogens. *Salmonella*, have the unique ability to colonise macrophages and have dedicated nitric oxide (NO) detoxification systems. There are three prominent metalloenzymes (HmpA, NorVW and NrfA) heavily researched in the literature for NO detoxification. Previous work suggested that more proteins are responsible for the nitrosative stress response with these being regulated by the nitric oxide sensitive transcriptional repressor, NsrR.

This study demonstrates a relationship between three putative tellurite resistance proteins regulated by NsrR (STM1808, YeaR and TehB) and NO detoxification. A Functional redundancy between these proteins was observed for anaerobic protection against NO and tellurite. Furthermore, this study identified that proteins responsible in NO protection such as HmpA and YtfE also provide resistance to tellurite during aerobic and anaerobic conditions, respectively. Tellurite resistant *Salmonella* strains were evolved by continued passage in this study that consequently had altered H_2O_2 resistance profiles and increased sensitivity to antibiotics. However, these strains were not significantly attenuated during macrophage survival or during the presence of NO *in vitro*. Additionally, the hypothetical protein YgbA, which has predicted roles in NO detoxification, was found to be important to *Salmonella* survival in macrophages. However, *in vitro* NO exposure with the NO donor deta NONOate only showed a role for anaerobic protection.

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[Fe-S]	Iron sulphur cluster
°C	Degrees Centigrade
AMR	Antimicrobial Resistance
Cfu	Colony Forming Units
Cm	Chloramphenicol
dH_2O	Distilled Water
DNA	Deoxyribonucleic Acid
DNRA	Dissimilatory nitrate to ammonium
E. coli	*Escherichia coli*
FNR	Fumarate and Nitric Oxide Reductase Regulator
Fur	Ferric Uptake Regulator
g	Gram
GMMA	Generalised Modules for Membrane Antigen
H_2O_2	Hydrogen Peroxide
HmpA	Flavohaemoglobin
IBS	Inflammatory Bowel Syndrome
IFN-γ	Interferon gamma
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
iNTS	Invasive Non-Typhoidal *Salmonella*
Kan	Kanamycin
L	Litre
LB	Luria-Bertani medium
M	Mole
MDR	Multidrug resistance
mg	Milligram
mL	Millilitre
mM	Millimole
N_2	Dinitrogen Gas
N_2O	Nitrous Oxide
NH_4^+	Ammonia
NO	Nitric Oxide

NO_2	Nitrite
$NO_2^{\cdot}$	Nitrogen dioxide Radical
NO_3	Nitrate
NorR	Nitric Oxide Reductase Transcriptional Regulator
NorVW	Flavorubredoxin with associated NADH-oxidoreductase
NrfA	Cytochrome *c* Nitrite Reductase
NTS	Non-Typhoidal *Salmonella*
O_2	Oxygen
$O_2^{\cdot}$	Superoxide Radical
OD	Optical Density
$OH^{-\cdot}$	Hydroxyl Radical
$ONOO^-$	Peroxynitrate
P	Passaged
PAMP	Pathogen Associated Molecular Pattern
PBS	Phosphate Buffered Saline
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
Rpm	Revolutions Per Minute
S. Typhimurium	*Salmonella enterica* serovar Typhimurium
SCV	*Salmonella* Containing Vacuole
Sif	*Salmonella* induced filament
Sod	Superoxide dismutase
SPI	*Salmonella* Pathogenicity Island
TLR	Toll-like Receptor
TNF-α	Tumour Necrosis Factor alpha
TR	Tellurite resistant
TTSS	Type Three Section System
Vi	*S.* Typhi Virulence Capsular Polysaccharide
μ	Growth rate
μg	Microgram
μL	Microlitre
μM	Micromole

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Chapter 1: Introduction

1.1. General *Salmonella*

Salmonella are gram-negative rod-shaped bacteria which belong to the *Enterobacteriaceae* family. These facultative anaerobes allow respiration using a range of electron acceptors which enables infection of diverse of organisms (Grimont et al., 2000). Within the *Salmonella* genus, there are two species, *S. bongori* and *S. enterica* (Tindall et al., 2005). *S. bongori* are predominantly known to infect cold-blooded hosts and are rarely the cause of human infections. Whereas *S. enterica* mainly cause disease in warm blooded animals (Chan et al., 2003). Six subspecies are present in *S. enterica* which are *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae*, (IV), *indica* (VI) with clinically relevant human pathogens residing in *enterica*. There are currently over 2600 serovars (Shi et al., 2015) within these subspecies which have been classified using a scheme generated by White-Kauffmann-Le Minor (WKL). Distinctions are made by using differences in somatic (O), flagellar (H) and capsular (K) antigens present on their outer membrane (Brenner et al., 2000). As Figure 1 highlights, these serovars are typically grouped by their ability to cause typhoidal or non-typhoidal infections. Generally, non-typhoidal *Salmonella* (NTS) serovars have a wide range of hosts that they are able to infect but there are exceptions (Andino and Hanning, 2015). For example, *S*. Pullorum causes Pullorum disease in chicks. This is an acute systemic disease with a high fatality rate and can be transmitted to other hosts easily (Barrow and Neto, 2011). Whereas *S*. Gallinarum primarily generates septicaemic infections in adult poultry named fowl typhoid fever (Hong et al., 2013). These host specific serovars can often result in asymptomatic carriers which allow the disease propagation in poultry (Kogut and Arsenault, 2017). Alternatively, *S.* Typhimurium is noted for its abilities to cause gastroenteritis in humans but also possessing the ability to cause a systemic typhoid-like infection in mice. While *S.* Dublin is specialised for causing respiratory infections and diarrhoea in calf's less than 12 weeks old. Following gastrointestinal colonisation, the infection can migrate through the bloodstream and progress to bacteriaemia. Those that survive such infection into adulthood can become chronic carriers which shed *Salmonella* in faeces thereby infecting new hosts (Nielsen, 2013). Conversely, typhoidal serovars such as *S.* Typhi and *S.* Paratyphi are host restricted and are specialised for causing disease in humans (Feasey et al., 2012, Uche et al., 2017).

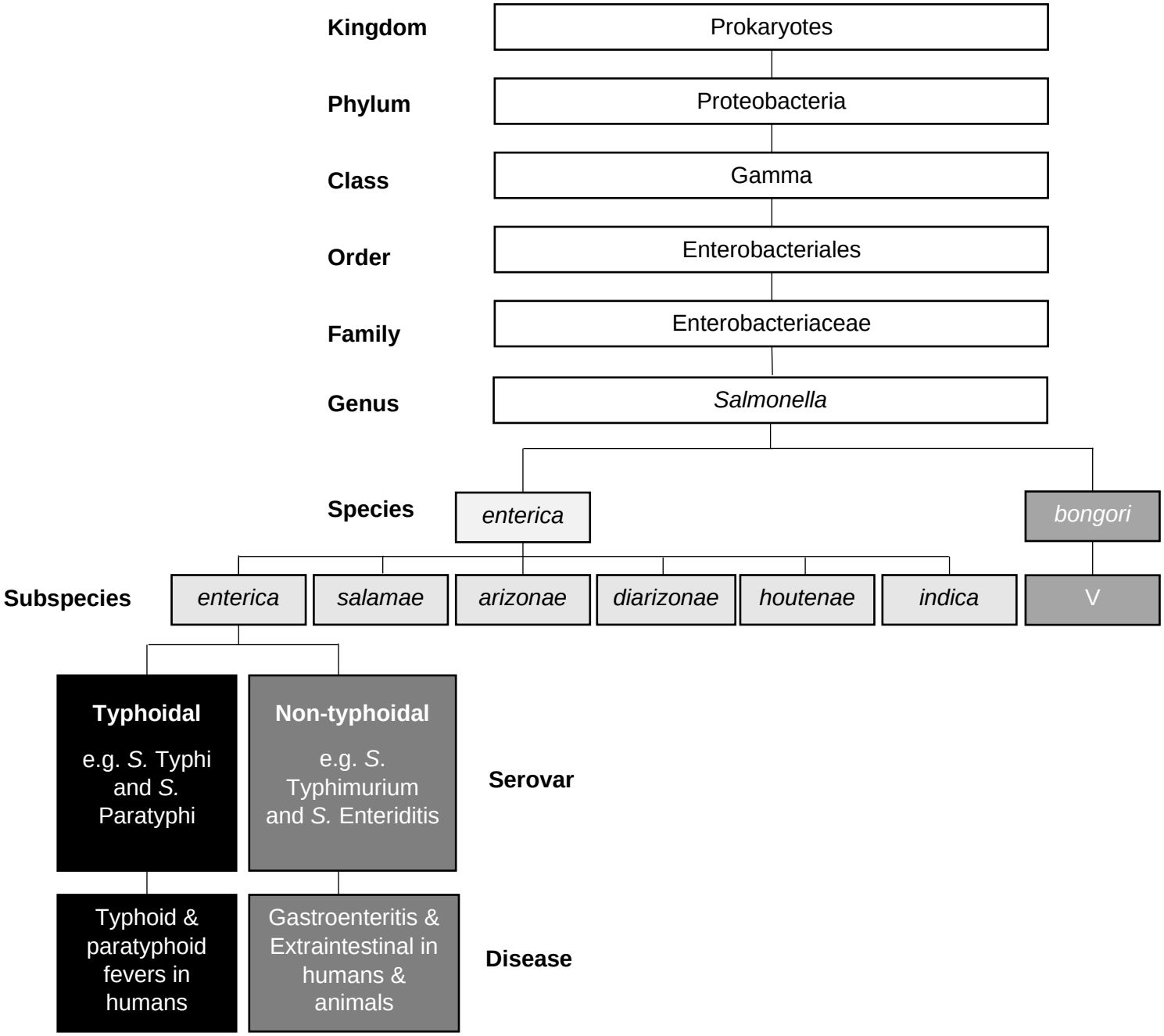


Figure 1. *Salmonella* nomenclature dendrogram detailing species, subspecies and serovar distinction.
The *Salmonella* genus contains *S. bongori* and *S. enterica*. Within *S. enterica* are six subspecies which contain numerous serovars that are distinguished by the disease caused by the pathogen (Tindall et al., 2005, Chan et al., 2003).

1.2. Infection route and contamination

Salmonella can be found within the food chain ranging from meat, fruit, vegetables, eggs and dairy products (Arnold et al., 2014, Cox et al., 2011, Foley and Lynne, 2008, D'Amico et al., 2008). Subsequently, the food industry have employed measures to ensure detection and elimination of enteric pathogens among others (Arroyo et al., 2012). The various range of contamination of foodstuffs displays how diverse and severe *Salmonella* infections are for both developing and westernised societies. Infections usually occur orally, through consumption of contaminated produce or water (O'Donnell et al., 2015) but they can also be acquired directly or indirectly with contact of infected animals or their faeces (Magwedere et al., 2015). Therefore, infections can be more prevalent in overpopulated societies which lack sanitation and hygiene procedures (Breiman et al., 2012).

Salmonella faces numerous stressful conditions whilst in the environment and after orally infecting the host they must withstand extreme low stomach pH, bile salts, high osmolarity and low oxygen in the intestines (Álvarez-Ordóñez et al., 2012). Typically, the bacterium invades epithelial cells or M-cells in Peyer's patches in the small intestine where macrophages can take in the *Salmonella* by phagocytosis within the terminal ileum (Jones et al., 1994), without this trait the *Salmonella* would be avirulent (Penheiter et al., 1997). Furthermore, they must outcompete commensal bacteria within the gut, then invade the intestinal epithelium and macrophages (Álvarez-Ordóñez et al., 2012). The bacterium can then replicate within a secure *Salmonella* containing vacuole (SCV) (Gorvel and Méresse, 2001) and become systemic by the transportation of infected macrophages to other organs, in the case of *S.* Typhi or Paratyphi (Richter-Dahlfors et al., 1997, Forest et al., 2010).

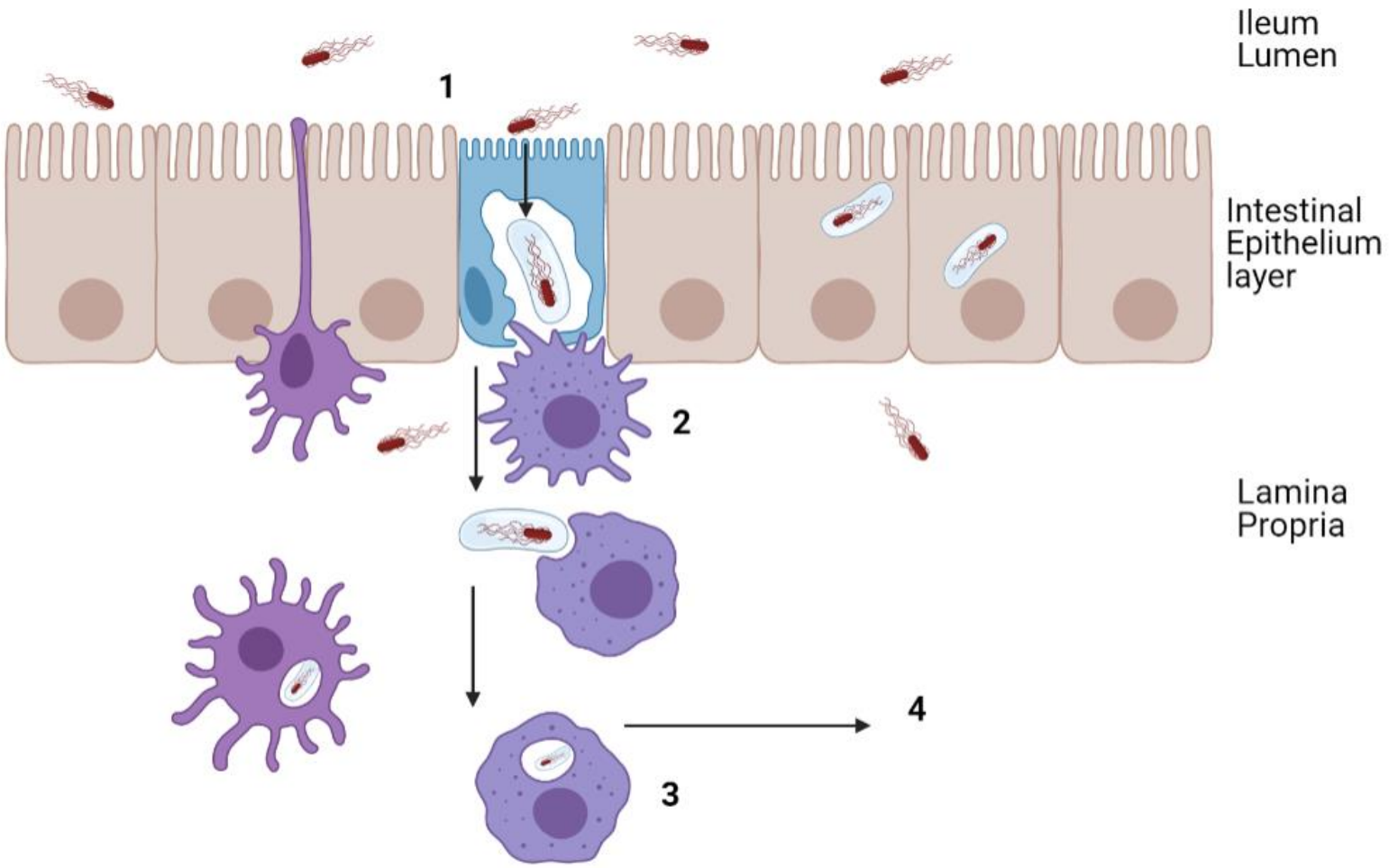


Figure 2. *Salmonella* infection route through epithelial (beige) or M-cell (blue).
1) adhesion an invasion of bacterium and the formation of the *Salmonella* containing vacuole (SCV). 2) activated macrophage and dendritic cell recruitment. 3) phagocytosis of SCV or escaped *Salmonella* in the lamina propria. 4) systemic dissemination to the liver and spleen. Inspired by (Knodler and Elfenbein, 2019) made using Biorender.

1.3. Incidence and disease

There are two main disease types associated with *Salmonella* infection which are systemic typhoid fever and gastroenteritis (Andino and Hanning, 2015). As reported by Majowicz et al. (2010) The global incidence of NTS cases in 2010 were estimated to be 98.3 million with 155,000 annual deaths. This has substantially increased to 153 million cases with contrastingly lower levels of fatality (only 57,000 annually) (Healy and Bruce, 2020). Whereas 21 million cases of typhoid fever were reported annually with 200,000 mortalities (Marchello et al., 2019). These figures are merely estimations, as many cases with milder symptoms go unreported or misreported as other enteric infections. Since the discovery of invasive NTS (iNTS), the global burden was estimated at 3.4 million cases with 681,316 deaths reported in 2010 (Crump and Heyderman, 2015, Marks et al., 2017). A 7-year surveillance study conducted by Hadler et al. (2020), found 91% of *Salmonella* infections were domestically acquired and children under 5 had the highest risk of infection followed by the over 65s. They discovered an increasing incidence of *Salmonella* infections with higher poverty levels. However, the paper stated limitations occur with these types of studies as not all Salmonellosis cases are laboratory confirmed due to the self-limiting nature of the illness. Furthermore, reporting may be limited especially in countries like the US where treatment requires payment which may not be an option for low-income families.

Gastroenteritis or Salmonellosis is typically less harmful, and a more common infection caused by non-typhoidal *Salmonella* (NTS) in humans and other animals. It is usually a self-limiting infection that does not require antibiotic intervention unless in young, elderly, or immunocompromised individuals (Gordon, 2008). Onset of symptoms can occur within 8-72 hours of exposure and include diarrhoea, vomiting, intestinal cramps or constipation and these symptoms can last up to 5 days (Gal-Mor, 2018, WHO and FAO, 2002). The cost of treating NTS infections are relatively low, but the economic burden has been estimated at 3 billion Euros in Europe alone (Országh et al., 2021). Furthermore, patients with previous NTS infections have been seen to develop chronic sequelae such as inflammatory bowel disease (IBS), ulcerative colitis and reactive arthritis (Beltran-Fabregat et al., 2006). This can cost the NHS up to £224,000 annually (Esan et al., 2020, Gal-Mor, 2018). A study by Esan et al. (2020) suggests that up to 80% productivity losses occur by workers as a consequence of taking time off work. Supporting the statement that NTS infections are a major health and economic burden to societies.

Enteric typhoid and paratyphoid fever is a much more severe infection. Symptoms of the disease include diarrhoea, blood or mucus filled stools, vomiting, fever, weight loss, confusion, inflammation of spleen and liver. Ultimately perforation of the intestines can occur which can result in sepsis and death by organ failure (Klotz et al., 1984, Gal-Mor, 2018). In the

unfortunate circumstance where no treatments are available the infection can be fatal. Enteric fever requires more vigorous treatment with intravenous antibiotics. The mean cost of illness for typhoid fever for individual patient $157.47 in Africa (Riewpaiboon et al., 2014), but can be as expensive as $432 per hospitalised patient in Indonesia (Poulos et al., 2011). This is much steeper because of longer hospitalisation periods, cost of antibiotics and cost of vaccination where this can be done. Uniquely, 2-5% patients with previous typhoidal infections can become chronic carriers of *Salmonella* that reside as biofilms in the gall bladder and are shed through faeces that can infect new hosts (Gonzalez-Escobedo et al., 2011, Gunn et al., 2014).

The symptoms of iNTS can include common gastro-intenstinal problems, but they can also be much more severe and can result in bacteraemia, meningitis, bone and joint infections (Feasey et al., 2012). This disease type has been discovered more recently and are distinguished by uniquely generating systemic infections in hosts with weakened immune systems (Uche et al., 2017). There is a problem with iNTS infections in high-risk groups such as HIV positive individuals because *Salmonella* exploits host defects such as dysregulated cytokine production, weaker gut mucosal layer and dysfunctional serum mediated elimination (Feasey et al., 2012). This has meant outbreaks of iNTS are endemic in regions with high HIV or malaria caseload such as in Africa (Marks et al., 2017). The decrease in transmission and better treatment for HIV and malaria has led to fewer cases of iNTS (Mackenzie et al., 2010).

1.4. *Salmonella* pathogenesis of infection

Salmonella possess many characteristics such as gastrointestinal epithelial adhesion, invasion, replication and survival in host cells which enable pathogenicity. They require virulence factors in order to manipulate the host immune system (Fàbrega and Vila, 2013). A number of these are located on highly conserved gene clusters noted as *Salmonella* pathogenicity islands (SPIs). SPI-1 encodes a type three section system (TTSS) and is required for invasion of host epithelial cells (Eade et al., 2019). The TTSS acts as a microscopic syringe by attaching to non-phagocytic adjacent cells and delivering virulent effector proteins which can have vast consequences for the receiving cell. SPI-1 Inv/Spa TTSS delivers effector proteins SopE, SopE2 and SopB which activate GTP binding proteins CDC42 and Rac1, consequently intercepting cell signalling pathways. Whilst SipA and SipC directly bind actin which facilitates polymerisation that mimics membrane ruffling allowing bacterial entry (Higashide et al., 2002, Lhocine et al., 2015). This process is similar to phagocytosis and allows the uptake of the bacterium (Figure 3). Then, SipA, SopB, SopD and SptP are used for *Salmonella* intravacuolar survival and replication (Ibarra and Steele-Mortimer, 2009).

Once inside the cell, *Salmonella* upregulate SPI-2 which is crucial for the maintenance of the *Salmonella* containing vacuole (SCV) (Gorvel and Méresse, 2001, Jennings et al., 2017). SPI-

2, which is controlled by the SsrAB two component system, is expressed upon low pH, phosphate and magnesium environmental ques (Löber et al., 2006). An Ssa TTSS is expressed from SPI-2 so that effector proteins SseL and SpvC can reprogramme cell signalling to block lysosomal fusion to the SCV. *Salmonella* induced filament (Sif) proteins are secreted and surround the vacuole with input from SseF and SseG proteins. The filaments can then extend using PipB and SopD2. SpiC is necessary for the secretion of translocon proteins SseB and SseC required for TTSS delivery of effector proteins and has been suggested to have roles in SCV membrane trafficking which is vital for *Salmonella* virulence (Freeman et al., 2002, Uchiya et al., 1999) (Figure 4). An *in vivo* study conducted by Grant et al. (2012) demonstrated that the knockout of SPI-2 resulted in high bacterial proliferation within cells. Therefore, they stated the role of the SPI-2 TTSS was to mediate exit from cells to generate systemic infection.

Salmonella uses SodCI to avoid NAD(P)H oxidase-killing as this superoxide dismutase protects the bacteria from extracellular reactive oxygen species (Rhen, 2019). The inducible nitric oxide synthase (iNOS) is able to use RNS to elicit bactericidal effects (Waterman and Holden, 2003) which will be discussed later. The capability of *Salmonella* detoxification allows the bacterium to replicate freely and allows it to uniquely survive within hostile macrophages (Eriksson et al., 2003) (Ibarra and Steele-Mortimer, 2009) in order to spread to other parts of the body. In addition, effectors from SPI-1 stimulate the proinflammatory response by secretion of chemoattractant IL-8 and IL-1 which promotes migration of neutrophils which can phagocytose exported SCVs from the lamina propria (Lee et al., 2000). They can then be transported to the lymphatic system and bloodstream where *Salmonella* can harbour in the spleen and liver (Figure 2). At this point the *Salmonella* infection has become systemic which occurs in typhoid fever or iNTS infections (Knodler and Elfenbein, 2019).

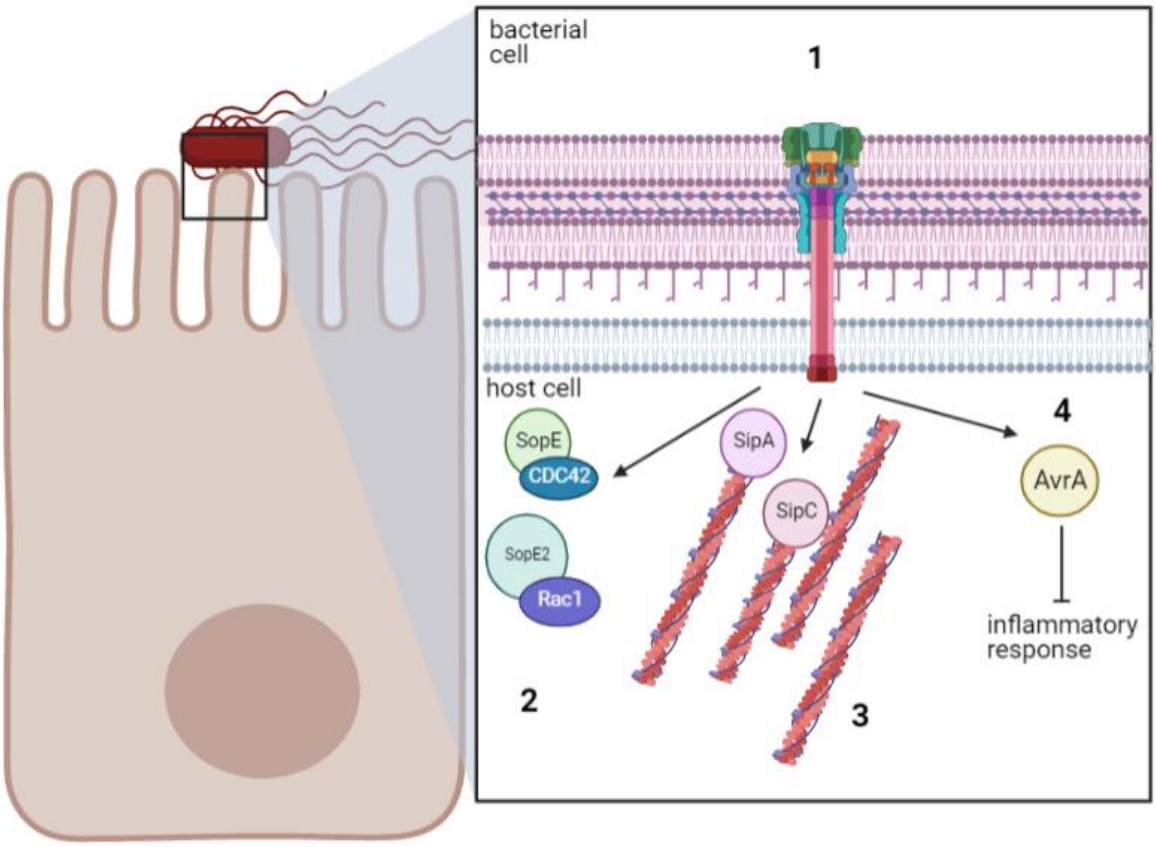


Figure 3. *Salmonella* adhesion and invasion in epithelial or M-cells.
1. Effector proteins are secreted from *Salmonella* into the host cytosol. 2. sopE and sopE2 interact with Rho GTP binding proteins such as CDC42 and Rac1 to impact host signalling. 3. SipA and SipC stimulate actin remodelling to allow bacterial invasion. 4. AvrA has anti-inflammatory effects by interfering with cell signalling. Made using Biorender. Inspired from (Waterman and Holden, 2003, Ibarra and Steele-Mortimer, 2009, Du et al., 2016, Brumell et al., 2001, Giacomodonato et al., 2014).

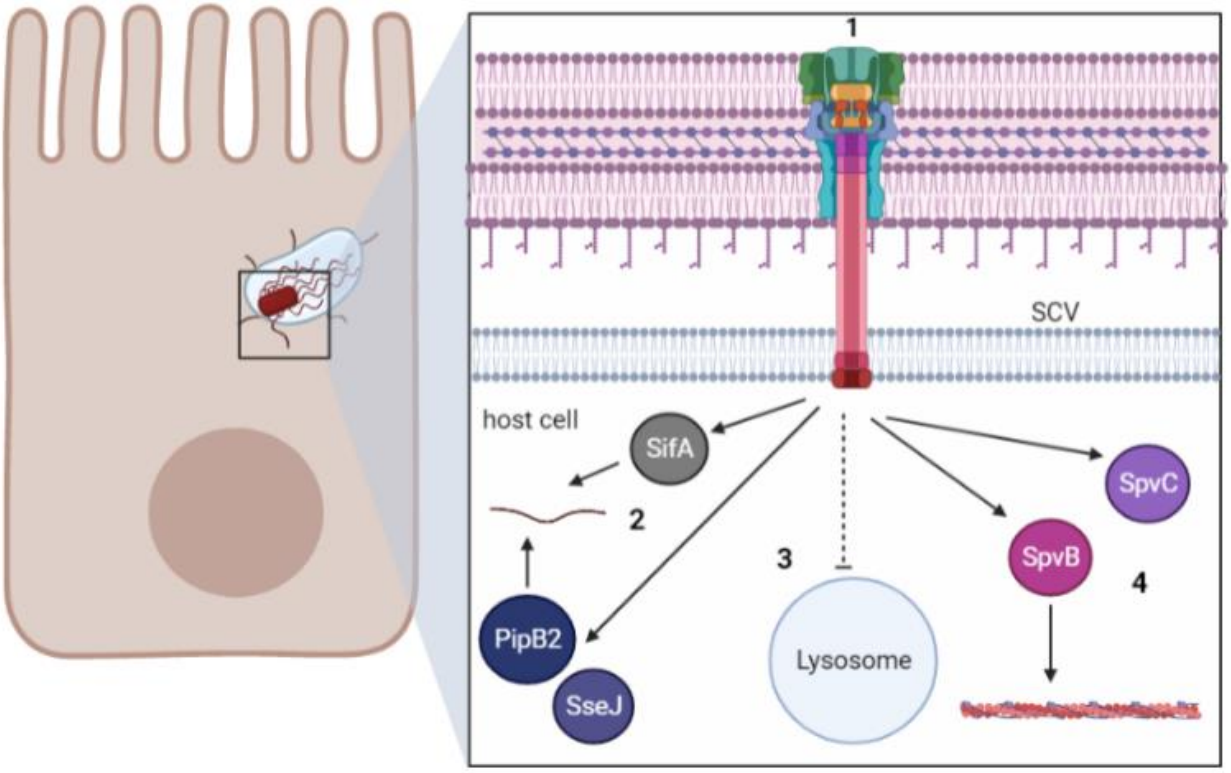


Figure 4. *Salmonella* persistence within cells.
1. Effector proteins are secreted from the SCV into the host cytosol. 2. SifA and PipB2 stimulate cytoskeletal remodelling and formation of *Salmonella* induced filaments (Sif) surrounding the SCV. 3. Lysosomal fusion is blocked through the interference with lysosomal production. 4. SpvB and spvC (located on the virulence plasmid) are cytotoxins released to the cytoplasm destabilising the cytoskeleton and inactivation of mitogen-activated protein kinases, p38, ERK1/2 and JNK in the host cell. Made using Biorender. Inspired from (Waterman and Holden, 2003, Ibarra and Steele-Mortimer, 2009, Du et al., 2016, Brumell et al., 2001, Giacomodonato et al., 2014).

The virulence plasmid in *Salmonella* Typhimurium is an IncF plasmid containing the principal genes *spvB* and *spvC* which can be seen in Figure 4. The expression of these genes is controlled by *spvR* which positively controls the *spvABCD* operon. Their gene products are delivered by the TTSS encoded on SPI-2 (Ibarra and Steele-Mortimer, 2009). The cytotoxin SpvB ADP-ribosylates actin, thus destabilising the cytoskeleton. It can also have an apoptotic effect on infected host cells (Browne et al., 2008). Whereas SpvC uses phosphothreonine lyase activity to disrupt mitogen-activated protein kinases such as Erk1/2, JNK and p38 which impact host cell signalling (Mazurkiewicz et al., 2008). Possession of this plasmid enables bacterial survival in neutrophils and macrophages by preventing autophagosome formation thereby interfering with host immune defences (Wu et al., 2016).

1.5. Current treatments and vaccines

Molecular diagnostics are crucial for the detection and clinical diagnosis of *Salmonella* as well as public surveillance. This is because accurate diagnosis of the disease can avoid unnecessary treatment with antibiotics (Vollaard et al., 2005). Antibiotics are still heavily relied upon for treating *Salmonella* infections in immunocompromised individuals and patients with typhoid fever with first line choices being cephalosporins, fluoroquinolones and macrolides (Guerrant et al., 2001). Cephalosporins work similarly to other β-lactam antibiotics by inhibiting peptidoglycan synthesis. Whereas fluroquinolones target DNA-gyrase or topoisomerases which thereby interfere with the reforming of DNA bonds during replication (Dowling et al., 2017). Macrolides inhibit peptidyl transferase activity or block the large ribosomal subunit which disrupts protein synthesis (Tenson et al., 2003).

According to a study conducted by Wang et al. (2019), 43% of antibiotic resistance in *S.* Typhimurium were seen in ampicillin, streptomycin, sulfonamides, tetracycline and chloramphenicol. Swine and bovine were the main reservoirs of these antibiotic resistance patterns due to the intensive usage of antibiotics inappropriately in rearing which is supported by a study conducted by Gupta et al. (2017) in *Escherichia coli*. The strains in Wang et al. (2019) were extremely sensitive to fluoroquinolones so they justified this is still an effective antibiotic to use against *S*. Typhimurium. Ciprofloxacin has become a frontline antibiotic to use to treat *Salmonella* infections and its effectiveness has been linked to the repression of SPI-2 gene expression consequently interfering with intracellular survival (Askoura and Hegazy, 2020).

Treating chronic carriers can be problematic as administering antibiotics could increase the duration of asymptomatic shedding without increasing the speed of bacterial clearance (Gunn et al., 2014). The issue resides in *Salmonella* biofilm formation which are naturally more resistant to therapies. However, a recent dual therapy study by Sandala et al. (2021) demonstrates that using anti-biofilm molecules JG-1 or M4 with ciprofloxacin greatly enhanced the disruption to pre-formed biofilms in a mouse model of chronic gallbladder carriage. Similarly, another study performed by Moshiri et al. (2018) identified a novel compound called T315, an integrin kinase inhibitor derivative, which could be used therapeutically for disruption of biofilms in the gall bladder of a chronic carrier.

The huge socioeconomic burden of *Salmonella* centres focus to develop more effective vaccinations for *Salmonella* and is an area of interest to many researchers. Vaccination programmes are more cost-effective prevention strategies (Guzman et al., 2006). The first vaccine that was developed for *S.* Typhi contained a heat-phenol-inactivated cell and became commercially available in 1916. The vaccine itself had between 51-88% efficacy and one dose

lasted the duration of 7 years. However, this vaccine was not widely useful due to unfortunate side effects such as headaches, fever and localised pain (Guzman et al., 2006). This vaccine would provide partial protection to *S*. Typhi intestinal colonisation, faecal shedding and systemic dissemination (Mastroeni et al., 2001). Whole cell inactivated vaccines are useful for stimulating adequate humoral antibody production, but they do not trigger sufficient levels of Th-1 cells or Th-2 responses for an adequate cellular response. Therefore, subunit vaccinations possibilities were explored such as the Vi purified non-denatured vaccine. Unlike the original vaccine, the Vi antigen vaccine displayed limited side effects and its only constraint was that boosters were required every 2 years. This protein was chosen as it is an important part of the *S.* Typhi capsule and is encoded on SPI-7 which is absent from *S.* Typhimurium (Helena, 2008). Sole use of Vi would gain between 55-75% efficacy and immunogenicity could be increased by the conjugation with toxins from cholera, tetanus, diphtheria or *Pseudomonas aeruginosa* porins (Klugman et al., 1996, Acharya et al., 1987). Newer Vi conjugate vaccines are more promising at providing a cost effective strategy to use in endemic regions (Lo et al., 2018).

Live attenuated vaccines are another alternative of preventing typhoid fever. Instead of components of the bacteria or a whole inactivated cell, this method used directed mutagenesis of key virulence proteins to attenuate *S.* Typhi growth. There are many advantages for using live-attenuated vaccines over previously developed ones because it can be administered orally by non-medical professionals, it can induce humoral, mucosal and cellular immune responses. One of the major limitations of developing these vaccines is the potential reversion back to virulence which is unlikely if there are multiple well-defined mutations. A good example of this is Ty21a which is an attenuated *S*. Typhi Ty2 strain that contains mutations in GalE and the Vi antigen (Formal et al., 1981). A trial conducted by Black et al. (1990) suggested the efficacy of a single dose would yield 29% or a double dose would be 30% more effective, but immunity only lasted for 2 years. There was development of another promising attenuated Typhi vaccine candidate containing *ΔaroC ΔssaVS* mutations. The M01ZH09 live oral vaccine was developed and proved to be an immunogenic and well tolerated following one dose in healthy volunteers with no evidence of bacteriaemia or prolonged shedding (Kirkpatrick et al., 2006).

There are currently vaccines in research and development for gastroenteritis caused by infections which have been reviewed in Tennant et al. (2016). There has been a particular focus on targeting multiple pathogen-associated molecular patterns (PAMPs) by using generalised modules for membrane antigen (GMMA) technology because it is relatively simple to generate, self-adjuvating and causes high immunologic potency at low costs. Other vaccine candidates are using recombinant or purified porins such as OmpC, OmpF, OmpD and

flagellin. These NTS vaccines would be beneficial to occupants of endemic regions where NTS is prominent and where there is high prominence of HIV especially as because of the increasing development of iNTS infections that are more difficult to treat. The usefulness can extend to westernised society to administer to the elderly as there is up to 50% fatality from NTS infections in this group. Currently there are many NTS vaccines which are in preclinical development which require more research and funding to put through clinical trials to assess their efficacy and safety.

Antimicrobial resistance (AMR) is becoming an alarming issue. Research has been ongoing to search for new drug targets. If increasing amounts of patients are being infected with AMR strains of *Salmonella,* they will become progressively more difficult to combat. The lack of vaccination has led to the dependence on partially effective antibiotics which has contributed to AMR in frontline treatments. It is crucial to continue researching drug targets to gain an understanding of how they can be used to develop new antimicrobials. Furthermore, research is pertinent for understanding virulence mechanisms that can become attenuated through mutagenesis that could present as effective vaccines. There has been interest in developing new therapies and the use of probiotics have been recognised as being highly efficacious with enteric pathogens (reviewed in Gut et al. (2018)). Another focus is looking at TTSS because they are conserved among gram negative bacteria so inhibiting these crucial virulence mechanisms could be attractive targets for treating *Salmonella* infections. For example, research conducted by Li et al. (2013) discovered that Cytosporone B and other analogues could successfully inhibit the inv/Spa TTSS. They weakened *Salmonella*'s ability to invade HeLa cells in an *in vitro* study. Furthermore, a collection of drugs called salicylidene acylhydrazides have been identified which block secretion of effector proteins from TTSSs (Negrea et al., 2007).

1.6. Immune response to *Salmonella*

Polymorphonuclear leukocytes are mature white blood cells with characteristic lobed nuclei and granule-containing cytoplasm. This includes eosinophils which have a role in inflammatory responses (Rothenberg and Hogan, 2006) and basophils which mediate immunoglobulin (Ig) E dependent responses (Falcone et al., 2000). Additionally, neutrophils which are essential for maintaining homeostasis within the intestine by protecting host cells from pathogenic microbes and minimising impact of dying cells (Fournier and Parkos, 2012, Amulic et al., 2012). Macrophages have the ability to produce reactive nitrogen and oxygen species (RNS/ROS) produced by iNOS and phagocyte oxidase (Phox), respectively. These harmful compounds are generated within the lysosome which fuses with the phagosome to form a phagolysosome and directed killing takes place by damage to DNA, protein and membrane

components. The expression of iNOS by macrophages is part of the pro-inflammatory pathway which generates a large quantity of NO. However, this process must be highly regulated as NO is also toxic to host cells (Aktan, 2004).

Once *Salmonella* has entered the host it elicits an inflammatory response mediated by the binding of lipopolysaccharides (LPS) in the cell membrane to complement proteins or toll-like receptor- 4 (TLR-4) with the co-receptor CD14 (Bernheiden et al., 2001). This and *Salmonella* specific antigens gain the attention of Th1 cells which stimulate the secretion of cytokines such as interferon-gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α) and Interleukin-17 (IL-17) that can recruit neutrophils (Kurtz et al., 2017). Neutrophils then secrete CAP18, cathepsin G and azurocidin chemoattractant which enables the second wave inflammatory response which entices macrophages to the mucosal area (Fournier and Parkos, 2012). As mentioned previously, *Salmonella* have the unique ability to survive within macrophages despite oxidative or nitrosative stressors and this is a crucial step in the infection process.

Salmonella SPI effector proteins are known to trigger inflammatory signalling in host cells (Lee et al., 2000). Examples include SipA, SopE, SopB, which as mentioned previously have an important role of epithelial invasion (Fàbrega and Vila, 2013). The AvrA protein has also been linked to anti-inflammatory responses in *Salmonella* infection. About 80% of *S. enterica* strains contain the *avrA* gene, but interestingly typhoidal strains lack this protein. Some researchers have suggested this links to their inability to elude epithelial defences that result in a more severe infection (Giacomodonato et al., 2014). Furthermore, they cause systemic infection due to their reliance on macrophage carriage to remain undetected from the host immune system.

Inflammasomes are key signalling platforms that form a crucial part of the innate immune system which protect intestinal epithelial cells from enteric infections. They allow a specialised form of cell death called pryoptosis which activates Interleukin-18 (IL-18). Moreover, caspase 1 from canonical inflammasomes is imperative for intestinal epithelial cell shedding to limit intracellular *Salmonella* (Han et al., 2019, Latz et al., 2013).

1.7. Nitric oxide toxicity and detoxification

Nitric oxide (NO) is a cytotoxic radical that can severely damage bacterial cell walls, proteins, transcription factors and DNA by interacting with thiols, ROS and Fe-S clusters (Arkenberg et al., 2011). Its mode of action has not always been fully understood and a study conducted by Jones-Carson et al. (2020) shows that NO prevents cellular division by inactivating 5'-monophosphate dehydrogenase and quinol oxidases in the electron transport chain. This

consequently depletes available nucleoside triphosphates which are required for energy dependent polymerisation of FtsZ. NO is highly reactive so it results in the production of other RNS such as peroxynitrite. This toxicity makes it an effective method for using against bacteria during the immune response.

Salmonella also encounter NO that is produced endogenously as a result of truncated denitrification whereby NO is reduced to N_2O or ammonium via the dissimilatory nitrite reduction to ammonium (DNRA) pathway seen in Figure 5 (Rütting et al., 2011, Jetten, 2008). Another method of dealing with endogenous NO is through oxidation to form nitrate (Lundberg et al., 2004) which is mediated through reacting with oxy-ferrous haemoglobin (Gow and Stamler, 1998). *Salmonella* encounter exogenous NO and other RNS through the infection process in the gastrointestinal tract. As previously stated, macrophages have the ability to produce NO in addition to other RNS through iNOS and this is done through the conversion of L-arginine to L-citrulline (Aktan, 2004).

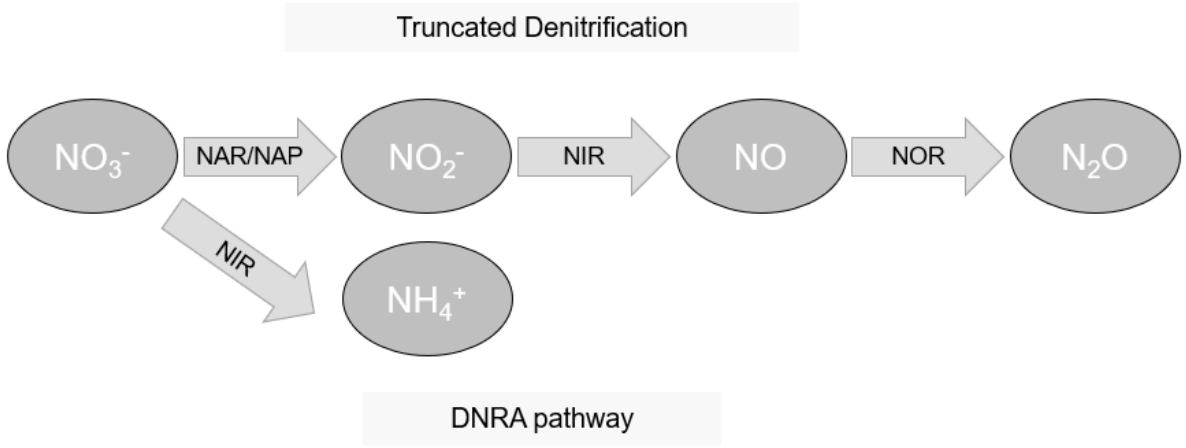


Figure 5. Truncated denitrification and dissimilatory nitrate reduction to ammonium (DNRA) pathways in *Salmonella*.
Nitrate (NO_3^-) is reduced to nitrite (NO_2^-) through membrane bound reductases (Nar) or within the periplasm (Nap). NO_2^- can then be reduced by nitrite reductase (NIR) and subsequent NO is reduced to nitrous oxide (N_2O) through nitric oxide reductase (NOR). Alternatively, in DNRA, NIR reduces NO_3^- into ammonia (NH_4^+) (Rütting et al., 2011, Lundberg et al., 2004).

Three metalloenzymes in particular have been identified within *Salmonella* to combat NO detoxification which are highlighted in Figure 6. These are flavohaemoglobin (HmpA), flavorubredoxin with associated NADH-oxidoreductase (NorVW), and cytochrome *c* nitrite reductase (NrfA) (Prior et al., 2009). They have all been shown to reduce NO *in vitro* but operate in different levels of oxygen presence. The anaerobic NrfA protein is only active in

anoxic or microoxic environments and works to convert NO to nitrite or ammonium (Mills et al., 2008). Whereas NorVW is able to reduce NO to nitrous oxide (N_2O). It is able to do this in either low oxygen or anaerobic conditions (Mills et al., 2005). HmpA primarily functions in high oxygen presence where it oxidises NO to nitrate, but it is also able to reduce NO to N_2O in anaerobic conditions (Gardner et al., 1998). A previous study conducted by Bang et al. (2006) demonstrates that HmpA is required for *Salmonella* survival and virulence in murine macrophages supporting that this protein is crucial for NO detoxification during pathogenesis.

The transcriptional repressor NsrR belongs to the Rrf2 family of transcriptional repressors. It contains an iron-sulphur [2Fe-2S] cluster which can sense NO through damage by nitrosylation. This consequently prevents the protein from binding to the promoter region upstream of the gene so it can no longer inhibit expression (Tucker et al., 2008). NsrR controls many genes including *hmpA*, *STM1808*, *yeaR-yoaG*, *tehB*, *ytfE, nrfA, hcp-hcr* and *ygbA* (Karlinsey et al., 2012). Due to the nature of the NsrR operon it is perceived that these genes function in NO detoxification which is true for *hmpA* and *nrfA*. However, there needs to be more research conducted on the other genes in *Salmonella*.

Many studies have been conducted in *E. coli*, a close relative to *Salmonella* and some responses overlap between the two bacteria. For example, the protein YtfE has recently been hypothesised to have a role in generating and repairing iron-sulphur clusters; therefore, *E. coli* deficient in *ytfE* were more sensitive to NO when grown anaerobically (Balasiny et al., 2018). In *Salmonella*, *ytfE* is known to be induced by NO through acidified nitrite, but its function remains elusive (Justino et al., 2005). Previous research by Wolfe et al. (2002) in *E. coli* suggests that the hybrid cluster protein (Hcp) is a hydroxylamine reductase. However, this was resolved to be a high-affinity NO reductase which functions to protect cytoplasmic proteins from NO-mediated damage through nitrite reduction to ammonia (Wang et al., 2016). YgbA is a small cytoplasmic protein which contains motifs from an aldehyde ferredoxin oxidoreductase domains 2 and 3 suggesting it may contribute to Fe-S cluster binding. However, it lacks in domains critical for molybdopterin ligand binding (Chan et al., 1995). The STM1808, yeaR and tehB proteins will be discussed in detail in the tellurite resistance section.

Other NO detoxification regulatory components include the fumarate and nitrate reductase regulator (FNR) which positively induces expression of NrfA in the presence of nitrate and nitrites (Gilberthorpe and Poole, 2008). FNR contains a [4Fe-4S] cluster which can be severely damaged from NO exposure (Balasiny et al., 2018). Additionally, the ferric uptake regulator (Fur) is mainly responsible for genes that control the uptake of iron, but it also has a role in repressing *hmpA* (Spiro, 2007, Justino et al., 2005). NorR upregulates *norV* expression which

can be switched on by various RNS including NO and particularly during macrophage infection around the NO burst (Mukhopadhyay et al., 2004, Eriksson et al., 2003).

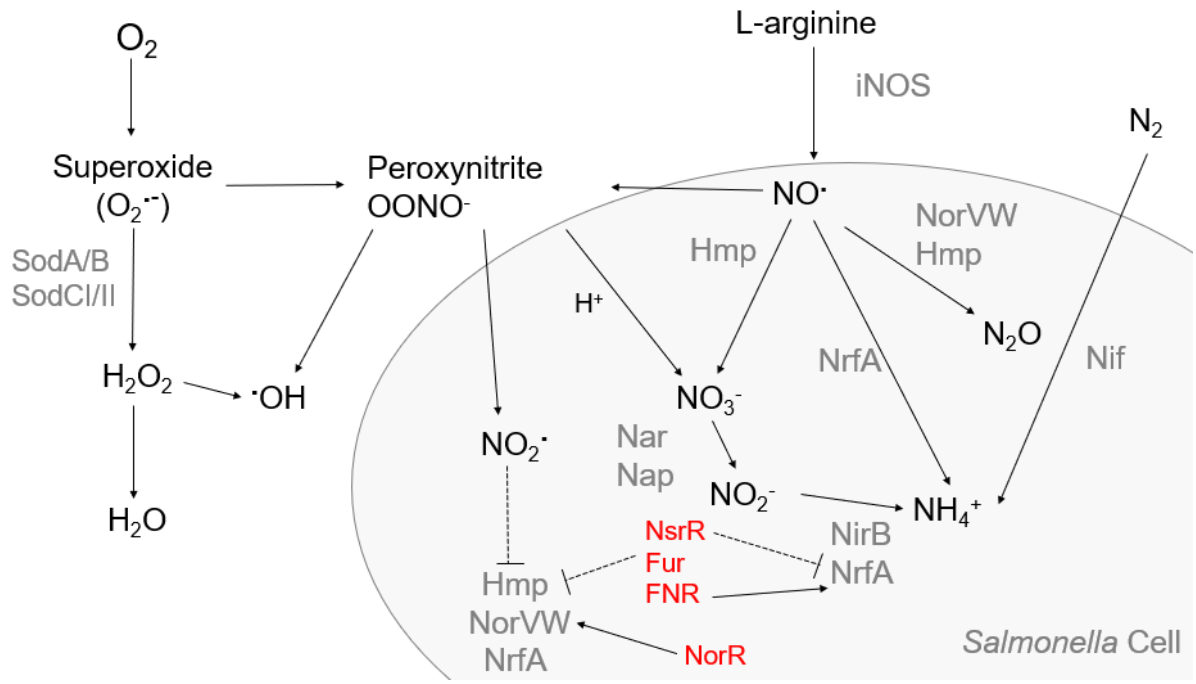


Figure 6. NO detoxification in *Salmonella* from exogenous and endogenous NO. Macrophages use inducible nitric oxide synthase (iNOS) to produce NO from L-arginine. This can form peroxynitrite ($OONO^-$) when combined with superoxide ($O_2^{\cdot-}$) and can even form other reactive oxygen and nitrogen species that damage pathogens. NorVW and Hmp can transform the toxic NO to nitrous oxide in anaerobic conditions or alternatively NrfA can form ammonia. When in aerobic conditions Hmp can convert the NO into nitrate (NO_3^-) which can be reduced to nitrite (NO_2^-) by Nar or Nap. NirB or NrfA can then be used to turn the nitrite into ammonia via dissimilatory nitrite reduction pathway. SodA, SodB, SodCI and SodCII are *Salmonella* superoxide dismutases that transform superoxide into hydrogen peroxide which can be turned into water or hydroxyl radicals via the Fenton reaction with liberated iron from broken Fe-S clusters. Detoxification methods employed by *Salmonella* are highly regulated. NsrR represses Hmp and NrfA when there is no NO present. Similarly, Fur can also inhibit Hmp expression. Whereas FNR positively enhances NrfA and NorR upregulates NorVW during exposure to RNS. In addition to this regulation Hmp, NorVW and NrfA can be inhibited by the production of nitrogen dioxide radicals. Generated using information from (Aktan, 2004, Mills et al., 2008, Prior et al., 2009, Gardner et al., 1998, Mills et al., 2005, Poole, 2020, Karlinsey et al., 2012, Rowley et al., 2012, McLean et al., 2010, Wang et al., 2018).

1.8. Tellurite toxicity and resistance

Tellurium (Te^0) is an element within group 16 among selenium, polonium, sulphur and oxygen, and these elements were classified as chalcogens with their compounds being referred to as chalcogenides (Fischer, 2001). Te^0 is a rare metalloid with four stable oxidation states including VI (tellurate), IV (tellurite), 0 (tellurium) and II (telluride). Despite the lack of abundance, there are rich pools of this element in deep sea hydrothermal vents, thermal springs, mine trailings and hypersaline environments (as reviewed by (Maltman and Yurkov, 2019)). These areas are not typically a niche *Salmonella* sp. occupy, but there is no evidence in the literature to suggest they do not encounter tellurium or otherwise. Research has been lagging compared to other heavy metals as tellurite has not been deemed essential for cellular processes (Maltman and Yurkov, 2019). Tellurite is a toxic oxyanion which can have numerous detrimental effects to bacteria that uptake it. The toxicity elicited by tellurite and ROS are highlighted in Figure 7 (Pérez et al., 2007).

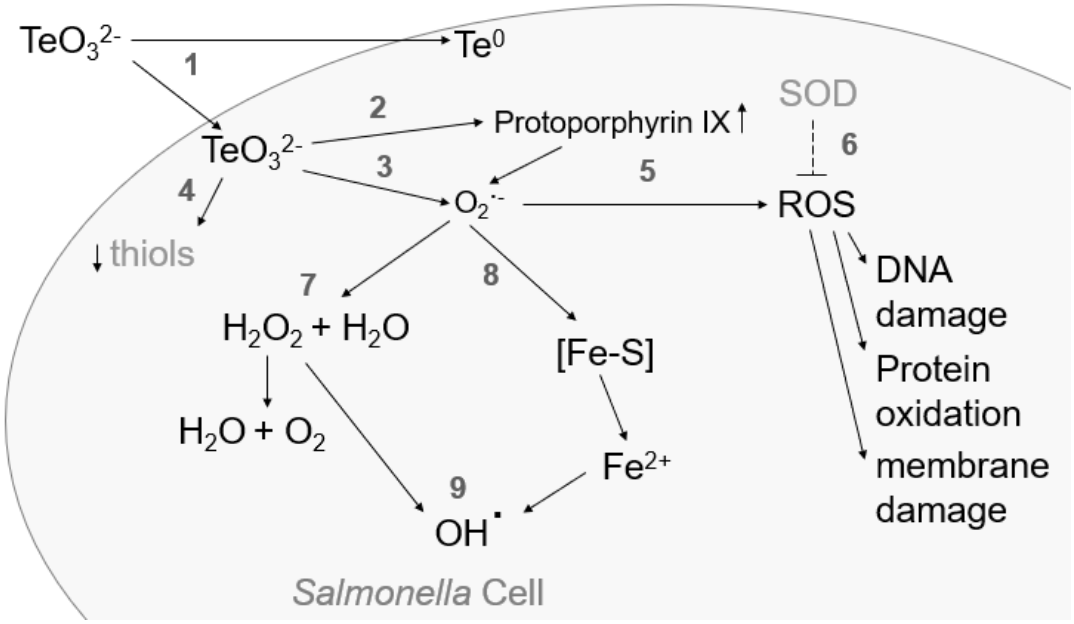


Figure 7. Tellurite is cytotoxic in bacterial cells by setting off a reactive cascade.
Tellurite (TeO_3^{2-}) enters the cell via porins or can be reduced to elemental tellurium (Te^0) through terminal oxidases (1). 2. Tellurite may interact with haem biosynthesis which as a result increases the amount of protoporphyrin IX which causes accumulation of superoxide (O_2^-) and other reactive oxygen species (ROS) 3. Superoxide formation occurs in the presence of tellurite. 4. Tellurite can cause the depletion of cellular thiols such as glutathione, thereby altering the cells reduction ability. 5. Other ROS are generation through radical reaction cascades, this can be prevented by superoxide dismutases (SOD) that can become upregulated by ROS exposure. 6. If ROS are not controlled they can cause DNA damage, oxidise proteins and even cause membrane damage that can be lethal. Further reactions occur with O_2^- by generation of hydrogen peroxide (H_2O_2) and harmless water, enzymes are present to detoxify H_2O_2 which lead to oxygen production (7). 8. Alternatively, O_2^- can damage iron sulphur clusters [Fe-S] which can liberate iron ions (Fe^{2+}) that can form damaging hydroxyl radicals ($OH^{\cdot}$) with H_2O_2 (9) (Pérez et al., 2007, Zannoni et al., 2007, Morales et al., 2017).

Tellurite resistance genes have been in existence in bacteria for a long time. For example, the *terD* gene from the *ter* operon has been used as a marker for tellurite resistance in *E. coli* (Lewis et al., 2018). Proteins encoded by the *ter* operon have also been investigated for virulence mechanisms of *Klebsiella* and *Yersinia* (Passet and Brisse, 2015, Ponnusamy and Clinkenbeard, 2015). The TehA and TehB (tellurite methyltransferase) proteins in *E. coli* were found to provide high levels of tellurite resistance but also providing resistance to antiseptics. Whereas KilA, TelA and TelB are encoded on the *kil-knr* operon and have been linked to roles in cell killing, plasmid replication and cryptic tellurite resistance in *E. coli* (Taylor et al., 1994, Goncharoff et al., 1991, Turner et al., 1994). *Salmonella* contain TehA and TehB orthologs, but they require more investigation. TxrR in *Bacillus* is a thioredoxin disulfide reductase which was essential for tellurite reduction and resistance (Yasir et al., 2020). Additionally, *cysK* which is the gene responsible for cysteine metabolism in *Bacillus stearothermophilus* transferred tellurite resistance when introduced into a deficient *Salmonella* strain (Vasquez et al., 2001). The importance of an *S*-Adenosylmethionine (SAM)-dependent methyltransferase domain has been highlighted in the literature as it was hypothesised that detoxification of tellurite could occur using these enzymes in a similar process to dealing with thiopurines (Cournoyer et al., 1998). These resistance systems require either strong upregulation or a large quantity of mutations present to display a tellurite resistance phenotype thereby suggesting that the primary function of such genes possess other metabolic roles (Taylor, 1999).

STM1808 is a *Salmonella* specific protein labelled as a putative cytoplasmic protein for tellurite resistance on the genome, but this has not been phenotypically tested. There is a conserved DUF1971 superfamily domain within the protein sequence which has an unknown function but is thought to have a role in tellurite resistance. It is not known to contain similar motifs to SAM-dependent N-methyltransferase which is considered important for other tellurite resistance proteins (Cournoyer et al., 1998). In a study conducted by Karlinsey et al. (2012) this protein was found to be controlled by the NsrR regulon. They suggested STM1808 was a zinc metalloprotein with important histidine residues (H32 and H82) which were required for NO resistance and zinc binding. They predicted the protein to have a role in the aerobic response to RNS and stated its importance in establishing a systemic infection in mice.

TehB is labelled on the *Salmonella* genome as a putative tellurite resistance protein which contains SAM binding motifs. TehB is a dimer that undergoes conformational changes to bind SAM and tellurite. Structurally both TehA and TehB contain three crucial cysteine residues which were confirmed to contribute to tellurite resistance as substituting these resulted in a phenotype sensitive to tellurite (Dyllick-Brenzinger et al., 2000). It is also known that TehAB provide resistance to tellurite by forming a functional electron transport chain and quinone pool

which alleviates ROS induced stress (Turner et al., 1995). Interestingly, TehA has been reported to mediate resistance against various antimicrobials including disinfectants in *E. coli* (Turner et al., 1997).

The YeaR protein was originally determined a hypothetical protein when the *S. enterica* genome was first sequenced (McClelland et al., 2001). It is now labelled as a putative tellurite resistance protein and research by Karlinsey et al. (2012) found that deletion of *yeaR* showed little to no effect upon NO addition compared to WT. Their experimentation does not resolve the role that YeaR plays in *Salmonella* for nitrosative stress or tellurite resistance. Therefore, further experimentation is required to find the role of this protein.

1.9. Research gap and project aims

Currently, a gap presents itself in the literature, which is discovering the purpose of *STM1808*, *tehB*, *yeaR* and *ygbA* for NO detoxification and tellurite resistance in *Salmonella*. This is because previous research has found these genes are upregulated during tellurite and NO exposure proving their importance and potential functional overlap. This project aims to uncover whether STM1808, TehB and YeaR proteins in *Salmonella* are required for tellurite resistance (chapter 3). The project aims to characterise STM1808, YeaR and TehB against nitric oxide stress by assessing the growth of deletion strains in the presence of a nitric oxide donor, Deta NONOate (chapter 3). Tellurite resistant strains will be evolved to enable phenotypic characterisation (chapter 4). Another aim is to characterise *ΔygbA* in various tellurite and NO growth conditions and in macrophage survival as this has not been previously investigated (chapter 5). This project will compare growth of SL1344 mutants lacking in nitrosative stress response proteins including *ΔhmpA*, *ΔnsrR*, *ΔyftE* and *Δhcp* and assess how these grow in tellurite compared to wildtype SL1344 (chapter 5). Furthermore, this project aims to discover if there is a correlation between *S*. Typhimurium tellurite resistance and nitrosative stress responses by the involvement of aforementioned proteins.

Hypothesis:

1. tellurite resistance proteins STM1808, TehB and YeaR will be crucial for protection against tellurite.
2. Evolved tellurite resistant strains will have altered phenotypic profiles which facilitate tellurite resistance as well as potential resistance or sensitivity to NO, H_2O_2, antibiotics and increased survival within macrophages.
3. The deletion of *ygbA* will attenuate the strains survival in tellurite, NO and survival within macrophages.

4. NO detoxification proteins such as Hcp, YtfE and HmpA are will mediate protection against tellurite as well as nitrosative stress.

Chapter 2: Materials and Methods

2.1. Strains and plasmids

Table 1. List of bacterial strains used in this research.
Kan^R refers to kanamycin resistance cassette, Cm^R refers to chloramphenicol resistance cassette.

Strain	Genotype	Reference
SL1344	*Salmonella enterica* serovar Typhimurium 4/74 *hisG*, *rspL*	(Hoiseth and Stocker, 1981)
ΔyeaR ΔSTM1808 ΔtehB	SL1344 *ΔyeaR*::Kan^R *ΔSTM1808 ΔtehB*::Cm^R	(Johnston, 2017)
ΔSTM1808	SL1344 *ΔSTM1808*	
ΔyeaR	SL1344 *ΔyeaR*	
ΔtehB	SL1344 *ΔtehB*	
ΔhmpA	SL1344 *ΔhmpA*::Cm^R	(Arkenberg, 2013)
ΔnsrR	SL1344 *ΔnsrR*	
ΔygbA	SL1344 *ΔygbA*::Cm^R	
ΔytfE	SL1344 *ΔytfE*::Kan^R	
Δhcp	SL1344 *Δhcp*	

2.2. Antimicrobials used

The following table outlines the antibiotic stocks used for experiments and which compounds were used to treat the strains with.

Table 2. Antimicrobial concentrations and preparations.

Compound	**Concentration**	**Preparation and storage**
Kanamycin from Sigma Aldrich	50 µg mL^{-1}	Prepared with dH_2O and filter sterilised then stored in 1 mL aliquots in the freezer.
Chloramphenicol from Sigma Aldrich	30 µg mL^{-1}	Prepared with ethanol and stored in 1 mL aliquots in the freezer.
Potassium tellurite (K_2TeO_3) Sigma Aldrich	5 mM stock	Prepared with dH_2O and filter sterilised then stored in the fridge. Dilutions used to generate subsequent concentrations in this study.
DetaNONOate from Cambridge Bioscience (supplied by Cayman Chemical)*	5 mM stock equivalates to 8 µM NO	Prepared with 10 mM NaHO and stored at -80 °C.
Hydrogen Peroxide (H_2O_2) from Sigma Aldrich	9 M and 1 M stocks	Diluted in dH_2O if necessary and stored in the refrigerator.
Gentamicin from gibco	50 mg mL^{-1} stock	Pre-prepared by manufacturer and stored at room temperature.
Polymyxin B from Sigma Aldrich	10 mg mL^{-1} stock	Weighed and prepared with dH_2O to desired concentration then filter sterilised.
Ceftriaxone from Sigma Aldrich	1 mg mL^{-1} stock	Weighed and prepared with dH_2O to desired concentration then filter sterilised.

*Deta NONOate is a nitric oxide donor which spontaneously liberates two moles of NO per mole of parent compound in a pH dependent manner. At a pH of 7.4, the donor has a half-life of only 20 hours at 37 °C and this can be up to 56 hours between the temperatures of 22-25 °C.

2.3. Media

Luria-Burtani (LB) media was made by dissolving 10 g NaCl, 10 g tryptone and 5 g yeast extract in distilled water (dH_2O) and autoclaved (121 °C, 15 psi for 15 minutes) to sterilise. LB agar plates were made with addition of 1.5% (w/v) to LB media, autoclaved and poured when needed.

Phosphate-buffered Saline (PBS) was made up by adding 4 tablets to a 500 mL Duran filled with 400 mL dH_2O and autoclaved to sterilise. Bacterial strains used on this study are listed in table 1. Media additions are listed in table 2.

2.4. Culturing and bacterial storage

For short term storage, bacteria were maintained on LB agar at 4 °C for up to two weeks supplemented with appropriate antibiotic(s). Stationary phase cultures were prepared from short term storage plates. A single colony was used to inoculate 10 mL LB broth (unless otherwise stated) supplemented with antibiotic where required and cultures were grown at 37 °C with aeration at 200-250 rpm overnight.

For long term storage, bacterial strains were stored at -80 °C in Microbank™ beads (ProLab Diagnostics) according to manufacturer's instructions.

Anaerobic cultures were made by filling 50 mL falcons to capacity, supplemented with appropriate antibiotic(s) and inoculated with a single colony from an LB agar plate. The top of the tube was then sealed with parafilm and incubated statically overnight at 37 °C. Cultures were washed twice with PBS prior to usage.

2.5. Aerobic growth

For manual aerobic growth, 250 mL conical flasks containing 50 mL media were inoculated with 1/100 (v/v) O/N culture as stated above and grown at 37 °C 250 rpm for 7 hours. Aliquots of 1 mL were extracted at hour intervals to measure the absorbance at 600 nm (OD_{600}) in the spectrophotometer using 1 mL of sterile LB as a reference. Due to the limitation of the spectrophotometer at readings above 1, the samples were diluted 1/10 (v/v) when necessary.

Automated aerobic growth curves were gained by using 24-well plates in a SpectraMax iD5 Microplate Reader with 10 seconds of agitation every 30 minutes before readings were taken during 37 °C incubation over 24 hours. This was set up by inoculating 1 mL LB in each well with 0.025 OD_{600} of O/N cultures.

2.6. Anaerobic growth

For manual anaerobic growth, 200 mL durans were filled with autoclaved media and inoculated 1/1000 (v/v) with washed anaerobic O/N cultures. The OD_{600} of O/N cultures was diluted accordingly and measured in a spectrophotometer to use as a zero reading. The durans were then sparged with N_2 for 10 minutes to mimic anaerobic conditions. These cultures were then incubated statically for 16 hours at 37 °C. Every 4 hours readings were taken using the spectrophotometer until 24 hours of growth.

Automated anaerobic growth readings were gained similarly as aerobic growth readings, but anaerobic O/Ns were used and 400 µL mineral oil was gently pipetted onto wells. The plate was then sealed with parafilm before placing in the SpectraMax iD5 Microplate Reader and incubated statically at 37 °C for 24 hours with readings every 30 minutes.

2.7. Minimum Inhibitory Concentration assays and percentage survival from tellurite treatment

LB was inoculated 1/1000 (v/v) with O/N culture which was grown as stated above. Cultures were treated with varied concentrations of potassium tellurite and grown in 37 °C incubation at 200 rpm for 12 hours. Final OD_{600} readings were taken by 1/10 (v/v) diluted measurements in a spectrophotometer.

The percentage survival of strains listed in table 1 were compared by growing O/N cultures and normalising these to an OD_{600} of 1. Subsequently, a serial dilution to 10^{-7} was conducted and these were spot plated onto select concentrations (0.1 µM, 0.2 µM and 0.5 µM). The colony forming units per 1 ml (Cfu/ml) was generated for treated and untreated plates using the calculation below and percentage survival was calculated to make the data more comparable.

$$\text{Cfu mL}^{-1} = \text{number of colonies X dilution factor X 10}$$

$$\text{Percentage survival} = \text{treatment Cfu mL}^{-1} / \text{control Cfu mL}^{-1} \text{ X 100}$$

2.8. Disc diffusion assay

From cultured bacteria (2.4.), 10 mL fresh LB were inoculated (10% v/v) and incubated for one hour at 37˚C, 200 rpm. LB agar (0.75% w/v), pre-warmed to ~60˚C, was aliquoted into sterile 5 mL Bijou tubes in 4 mL volumes and allowed to cool before the addition of 100 µL of the 10% subculture. Molten agar containing the culture was immediately poured over pre-set 1.5% (w/v) LB agar plates and left to set.

Sterile Whatman® antibiotic assay discs (6 mm) were aseptically placed onto pre-poured and set top agar plates and impregnated with 10 µL of the compound of interest (table 2). Plates were then incubated at 37˚C for a minimum of 12 hours and the zones of inhibition as area by mm^2 were recorded.

2.9. Gentamicin protection assay

Monocyte macrophages from a mouse leukaemia cell line (RAW 264.7) were maintained using DMEM media supplemented with 10% foetal bovine serum, 2 mM L-glutamine and placed in a 5% CO_2 incubator at 37 °C. Swabs of freshly streaked bacterial strains of interest (2.4.) were inoculated in PBS and OD_{600} measured. This was normalised to a dose of $1x10^7$ cells when inoculated 1:10 (v/v) into fresh DMEM. This allowed a multiplicity of infection of 10 bacterial cells per one macrophage which were seeded in a 12-well plate 24 hours prior to infection. Strains were allowed to infect cells for 1 hour before being washed twice with PBS and treated with kill media containing 100 µg mL^{-1} gentamicin. These were then incubated for a further hour or 22 hours. After washing the 24-hour infection plate a maintenance media was used containing 10 µg mL^{-1} gentamicin. After specified incubation periods the cells were subjected to two PBS washes and lysis using dH_2O containing 1% Triton X-100. Serial dilutions were then prepared for spot plating on LB agar to allow Cfu/mL calculations. This assay enables the quantification of bacterial survival within macrophages as the antibiotic gentamicin poorly penetrates eukaryotic cells.

2.10. Bioinformatic investigation into conservation of STM1808, YeaR and TehB

Conservation between STM1808, YeaR and TehB was assessed using NCBI resources using *Salmonella* Typhimurium LT2 whole genome (AE014613.1) to search for each amino acid sequences or gene names. Comparison genomes used were *S*. Typhi Ty2 (AE014613.1), *S*. Dublin (CP074226.1) and *S. bongori* 40:z35 (CP074592.1). Protein sequences were gained using the ExPASY translation tool and amino acid sequences were compared accordingly to *S.* Typhimurium using the EMBOSS water pairwise sequence algorithm to resolve percent identity. This software identified amino acid substitutions which could be used for further analysis on impacting protein functionality.

2.11. Statistics

All data was collected in triplicate to allow for statistical comparison which was performed using the Microsoft Excel XRealStats package. Analysis of Variance (ANOVA) was calculated on MICs and percentage survival studies or student's t-test was used (specified in Figure legends). Growth curves were compared using growth rates ($\mu = (\log_e 2)/ Td$) and doubling times ($Td = (\log_e 2)/\mu$). Significance threshold was denoted as $p<0.05$.

Chapter 3: Elucidating the roles of tellurite resistance proteins in nitric oxide detoxification

3.1. Introduction

As introduced in Chapter 1, *Salmonella* produce RNS through nitrate or nitrite metabolism in a process termed truncated denitrification (Rowley et al., 2012). On top of this, *Salmonella* relies on survival within macrophages which produce RNS through iNOS during infection (Lee et al., 2000). Both of these processes encompass an interaction with cytotoxic NO which can interact and negatively impact cellular metabolism, DNA, proteins, and the cell membrane (Arkenberg et al., 2011).

Designated NO detoxification systems are used to overcome these challenges. These include the anaerobic flavorubredoxin with associated NADH-oxidoreductase (NorVW), the aerobic and anaerobic functioning flavohaemoglobin (HmpA) and strictly anoxic cytochrome *c* nitrite reductase (NrfA) (Prior et al., 2009). NorVW is able to reduce NO and form N_2O and this system is positively induced by the transcriptional regulator NorR (Mills et al., 2005). Whereas NrfA can generate nitrite or ammonium from NO and is directly controlled by the repressor NsrR (Karlinsey et al., 2012). NrfA relies on the anaerobic respiratory activator FNR for appropriate transcription (Mills et al., 2008). HmpA is tightly regulated as it is able to function with and without the presence of oxygen whereby NO is oxidised to nitrate and reduced to N_2O, respectively (Gardner et al., 1998). For this reason, HmpA is repressed by NsrR (Karlinsey et al., 2012), FNR and by Fur (Justino et al., 2005) due to altered iron levels that cells experience in the presence of NO.

As stated in Chapter 1, NsrR is a transcriptional regulator which is a member of the Rrf2 family. It contains an Fe-S cluster that actively senses NO by undergoing reversable nitrosylation. This prevents dimer formation of the protein, thereby inhibiting DNA binding and uncovering the promoter region to allow transcription of NO detoxification genes (Tucker et al., 2008). FNR functions similarly to NsrR as it also contains a Fe-S cluster which is responsive to the presence of O_2 and NO (Khoroshilova et al., 1997, Constantinidou et al., 2006).

The transcriptomic profile of *E. coli* generated by Justino et al. (2005) stated that *tehB* and *yeaR* genes were upregulated when anaerobic cultures were exposed to 50 μM NO. Furthermore, transcriptional analysis through a microarray conducted by the Rowley Lab identified *STM1808*, *yeaR* and *tehB* genes to be significantly upregulated post exposure of *Salmonella* Typhimurium SL1344 to 40 μM NO (Arkenberg, 2013). These genes share a transcriptional regulator, NsrR which is NO sensitive, and controls other genes which are important in NO detoxification strategies. Further investigation is required in order to resolve whether STM1808, TehB and YeaR tellurite resistance proteins play a critical role in NO

detoxification. Therefore, this chapter will focus on the conservation of STM1808, YeaR and TehB in *Salmonella* across serovars and their potential involvement in NO detoxification by assessing attenuation in growth during exposure of NO in deletion mutants.

Tellurite is toxic and its bactericidal action has been debated for years but it has strong oxidative properties, so it is thought to deplete ATP stores and oxidise cellular thiols resulting in altered reduction potential in cells. It is known to interfere and bind to cysteine residues and cysteine metabolism has been established as important as defects in this process has led to tellurite resistance in *E. coli* (Turner et al., 1999). Cysteine is an essential amino acid required for synthesis of glutathione which serves as a ROS scavenger (Smirnova and Oktyabrsky, 2005) giving this vital tripeptide an important role in detoxifying tellurite. Tellurite can also bind to selenium, an element similar to tellurium, which can be found in some proteins and these elements can even replace sulphur groups, subsequently impacting protein function (Taylor, 1999, Ba et al., 2010). Despite the similar biochemistry between tellurite and selenite, tellurite is ~1000 more toxic due to the rapid loss of thiols (Turner et al., 2001). This may be because tellurite stimulates reactive oxygen species (ROS) such as hydroxyl radicals via the Fenton reaction ($TeO_3^{2-} + 3\ H_2O + 4\ e^- \rightarrow Te^0 + 6\ OH^-$) described by Turner et al. (1999) which require detoxification (Lohmeier-Vogel et al., 2004). This can impact non-resistant and highly resistant microbes, proving that tellurite is a large nuisance for bacteria (Maltman and Yurkov, 2019).

As stated in Chapter 1, there are five mechanisms of tellurite resistance which are reviewed by Taylor (1999) and these have been found to be both chromosomal and plasmid derived. These include TehB tellurite methyltransferase and TehA which provide resistance to tellurite when overexpressed in a high copy plasmid as well as antiseptics and disinfectants which employs an efflux pump method (Turner et al., 1995, Turner et al., 1997). Transcription of *tehAB* is negatively regulated by NsrR which as mentioned earlier becomes deactivated during exposure to NO (Karlinsey et al., 2012). The *ter* operon is typically found on IncHI2 and IncHII conjugative plasmids and provide resistance to tellurite, bacteriophages and colicins (Taylor, 1999, Whelan et al., 1997). KilA is a 28-kDa protein which was originally found on the IncP α-plasmid RK2Ter with other genes involved in plasmid replication. TelA is hydrophilic like KilA and is 42-kDa whereas TelB is hydrophobic and 32-kDa which means it is likely to be membrane bound. A Ser-Cys missense mutation in TelB was responsible for tellurite resistance in the RK2 plasmid (Walter et al., 1991). The cysteine synthase protein CysK has been determined to transfer tellurite resistance from *Bacillus stearothermophilus* to *E. coli* and *S*. Typhimurium (Vasquez et al., 2001). Disulphide bond formation in periplasm proteins DsbA and DsbB have been determined by Turner et al. (1999) to be important in basal resistance to tellurite. Zannoni et al. (2007) described four ways that tellurium oxyanions could be dealt with including reduction using glutathione, catalytic reduction by periplasmic or cytoplasmic

oxidoreductases, a reduction-oxidation reaction involving the iron siderophore PDTC and direct or indirect reductions from electrons siphoned from the membrane bound respiratory chain.

The genes *yeaR*, *STM1808* and *tehB* have been labelled for tellurite resistance in the *Salmonella* genome. *STM1808* is *Salmonella* specific and requires more research. Whereas homologues of *tehB* are found in *E. coli* and are accordingly more investigated in the literature (Turner et al., 1995). There is little exploration into *yeaR* apart from regulation studies which have been conducted in *E. coli* and *Salmonella* (Justino et al., 2005). These genes are controlled by the transcriptional repressor NsrR which controls other NO detoxification genes including *nrfA*, *hmpA*, *ygbA*, *ytfE*, *hcp-hcr* (Karlinsey et al., 2012). These genes sharing the same transcription factor suggests that the genes possess a degree of functional overlap as gene clusters tend to congregate similar biological processes.

Due to the proposed mode of action for tellurite toxicity within bacteria, it would not be implausible for there to be a link between nitrosative stress responses and tellurite resistance in *Salmonella*. This hypothesis has not yet been tested and this gap in the research could be filled with data collected in this study. Other metals have been shown to be of importance during NO stress. For example, research by Yousuf et al. (2020) highlighted the use of manganese for bacterial recovery following exposure to NO. Similarly, zinc has previously been investigated in *Salmonella* as the metal can be mobilised from metalloproteins upon exposure to NO which requires efflux out of the cell (Frawley et al., 2018).

The rarity of tellurite in the environment means the likeliness of *Salmonella* exposure is narrow. The question remains what the purpose of keeping such genes labelled for tellurite resistance unless they can be useful to *Salmonella* pathogenesis. This is because they could have become pseudogenes or lost during evolution instead of being retained. Furthermore, it warrants further investigation to uncover the prominence of these proteins that are labelled for tellurite resistance in other serovars that have genetic differences to Typhimurium.

3.2. Chapter Aims

As summarised above, the tellurite resistance genes *yeaR*, *tehB* and *Salmonella* specific *STM1808* share regulation by NsrR with various genes that have been determined crucial for NO detoxification. It is plausible for these genes to encode proteins which contribute to anti-nitrosative stress. Much research has previously been conducted on *E. coli*, which has limited the current understanding of these tellurite resistance proteins in *Salmonella* especially in the case of *STM1808*. Additionally, there is phenotypic work missing for *tehB and yeaR* deletion mutants in the presence of NO and all three genes in the presence of lower concentration of tellurite. It was hypothesised that *STM1808*, *yeaR* and *tehB* have a functional overlap for

tellurite resistance and NO detoxification. Furthermore, the prominence of these genes across different *Salmonella* serovars would support the importance of these tellurite resistance genes and the argument that they hold alternative functions that are beneficial to *Salmonella*.

This chapter aimed to:

1. Investigate the conservation of STM1808, YeaR and TehB tellurite resistance proteins across different serovars across *S. enterica* against *S. bongori*.
2. Determine the importance of tellurite resistance proteins for low concentrations of tellurite in the presence and absence of oxygen.
3. Assess exposure to NO for both aerobic and anaerobic growth conditions.

These aims were achieved by:

- Analysing differences in growth of deletion mutants lacking in *STM1808*, *tehB* and *yeaR* compared to WT SL1344 in aerobic and anaerobic conditions during concentrations of K_2TeO_3 ranging (0.1 μM – 5 μM) and deta NONOate (5 mM).
- Bioinformatic investigation using NCBI to assess if *S. enterica* Typhimurium copies of STM1808, YeaR and TehB are found among *S. enterica* Typhi, *S. enterica* Dublin, *S. bongori*.

3.3. Results

3.3.1. Deletion mutants grow similarly to wildtype under aerobic and anaerobic conditions in LB

Deletion strains were formed previously by Johnston (2017) which were generated through λ-red recombination. These strains were lacking in tellurite resistance genes, *yeaR*, *STM1808* and *tehB*. Single mutants were deficient in one of these genes, but a triple deletion strain denoted as *Δx3* was generated which lacked all three genes. Growth curves were conducted in rich LB media both in aerobic and anaerobic conditions which ensured all strains had no growth deficiency despite gene deletions. This increases the likelihood that phenotypic discoveries identified in this study are more genuine. From this graph it can be seen that slight differences in growth were observed but upon close examination of each strains growth rate these were not statistically significant aerobically (p=0.4, F_4=1.14) or anaerobically (p=0.4, F_4=1.09).

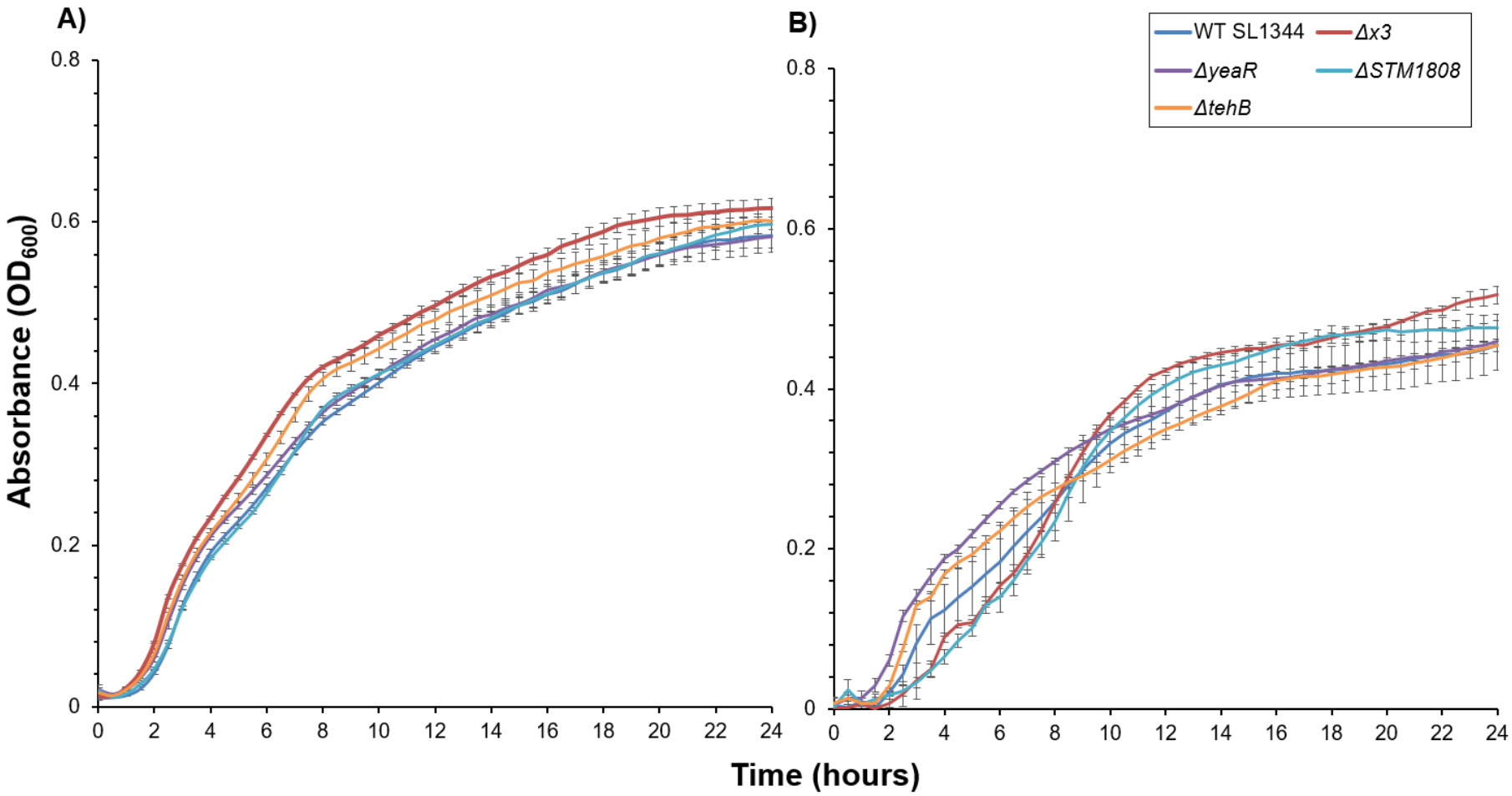


Figure 8. Similar growth is observed between wildtype SL1344 and deletion mutants in both aerobic (A) and anaerobic (B) conditions. Cultures were grown in 1 mL LB in a 24-well plate and grown over 24 hours with 30-minute interval readings of optical density taken at 600 nm. Data is comprised of three biological replicates with standard error bars plotted for each strain.

3.3.2. Tellurite resistance proteins are required for *Salmonella* survival during exposure to tellurite under aerobic or anoxic conditions

There is a lack of empirical evidence of tellurite resistance in *Salmonella* deficient in STM1808, YeaR and TehB in the literature. Previous work by Johnston (2017) identified a phenotype of 70% reduction in growth determined by OD in aerobic and 50% in anaerobic conditions with 5 μM K_2TeO_3 in WT SL1344. Additionally, a visible extended lag phase was observed aerobically in deletion mutants which was less prominent anaerobically. Taking these findings into consideration, it was evident that exploring the response of these strains in lower concentrations was required. This is because depletion of growth seen in WT was too extensive and not distinguished enough from deletion strains. Therefore, the addition of different concentrations ranging from 0.1-0.5 μM of tellurite were screened using the SpectraMax iD5 plate reader with various deletion strains as listed above.

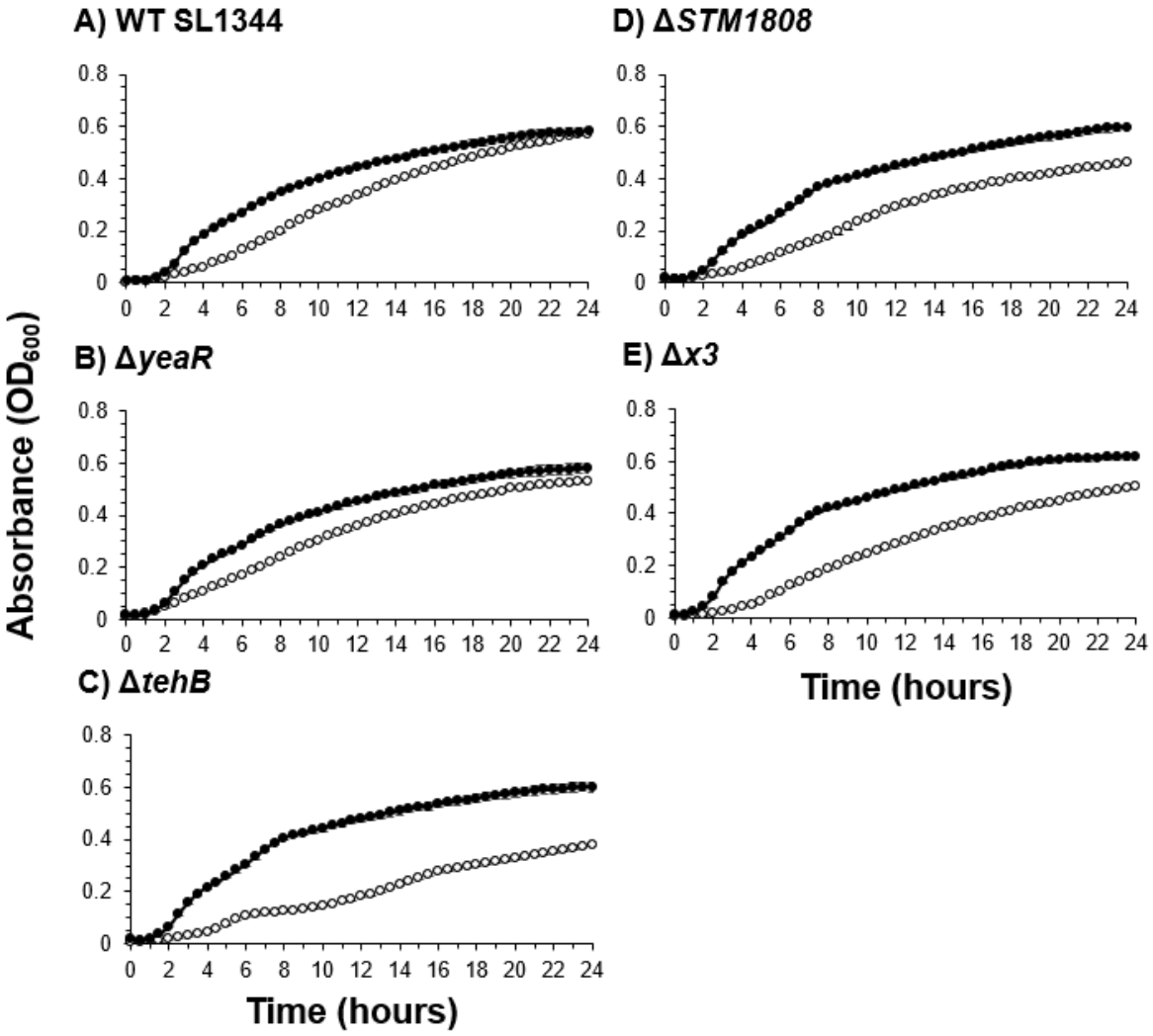


Figure 9. Deletions of *STM1808*, *tehB* and *yeaR* individually contribute to attenuation in the triple mutant during aerobic tellurite exposure.
Strains were growth with the concentration of 0.3 μM potassium tellurite (white circles) and without (black circles) in aerobic conditions in 1 mL LB. Readings of optical density at 600 nm were taken every 30 minutes over 24 hours. A) WT, B) *ΔyeaR*, C) *ΔtehB*, D) *ΔSTM1808*, E) *Δx3*. Data shows three biological repeats and standard error is plotted for each strain.

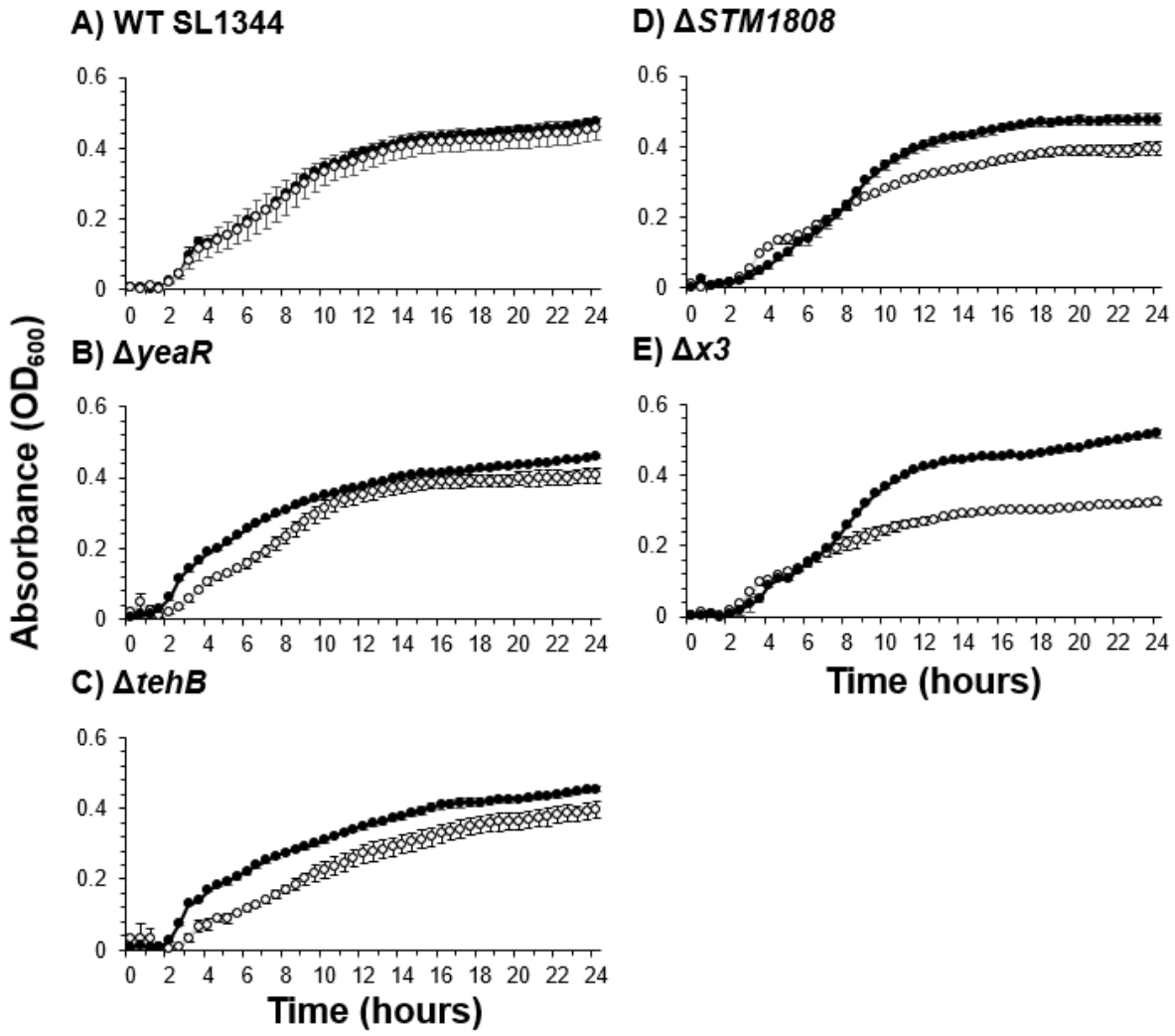


Figure 10. There is a functional redundancy in tellurite resistance proteins during anaerobic tellurite exposure.
Strains were growth with the concentration of 0.3 μM potassium tellurite (white circles) and without (black circles) in anaerobic conditions in 1 mL LB. Readings of optical density at 600 nm were taken every 30 minutes over 24 hours. A) WT, B) *ΔyeaR*, C) *ΔtehB*, D) *ΔSTM1808*, E) *Δx3*. Data shows three biological repeats and standard error is plotted for each strain.

The addition of tellurite reduced growth for all strains, but this was particularly enhanced in *ΔtehB*, *ΔSTM1808* and *Δx3*. WT experienced a reduction in growth rate of 48% aerobically and no difference in growth rate anaerobically compared to growth without potassium tellurite. The triple mutant experienced a 46% decrease in growth rate aerobically and this was enhanced lower to 51% reduced anaerobically. The decrease in growth rates of *ΔyeaR* was also 48% loss aerobically and no difference anaerobically which is the same as WT SL1344, which is a contrast to *ΔSTM1808* and *ΔtehB* that were reduced by 33% and 27% aerobically and 65% and no change anaerobically, respectively. Consequently, supporting previous work that TehB is crucial for tellurite resistance due to the dramatic reduction in growth which was similar to the triple deletion strain. Furthermore, STM1808 must have a key role which was more apparent during anaerobic growth as tellurite had a deeper impact to *ΔSTM1808* growth rate without the presence of oxygen.

Due to microoxic conditions and the tendency of biofilm formation occurring in the plate reader, results are less likely to give an accurate impression of the deleterious effects of tellurite. Under this presumption further batch cultures of WT SL1344 with *ΔyeaR ΔSTM1808 ΔtehB* were conducted aerobically with 0.2 μM and 0.5 μM potassium tellurite.

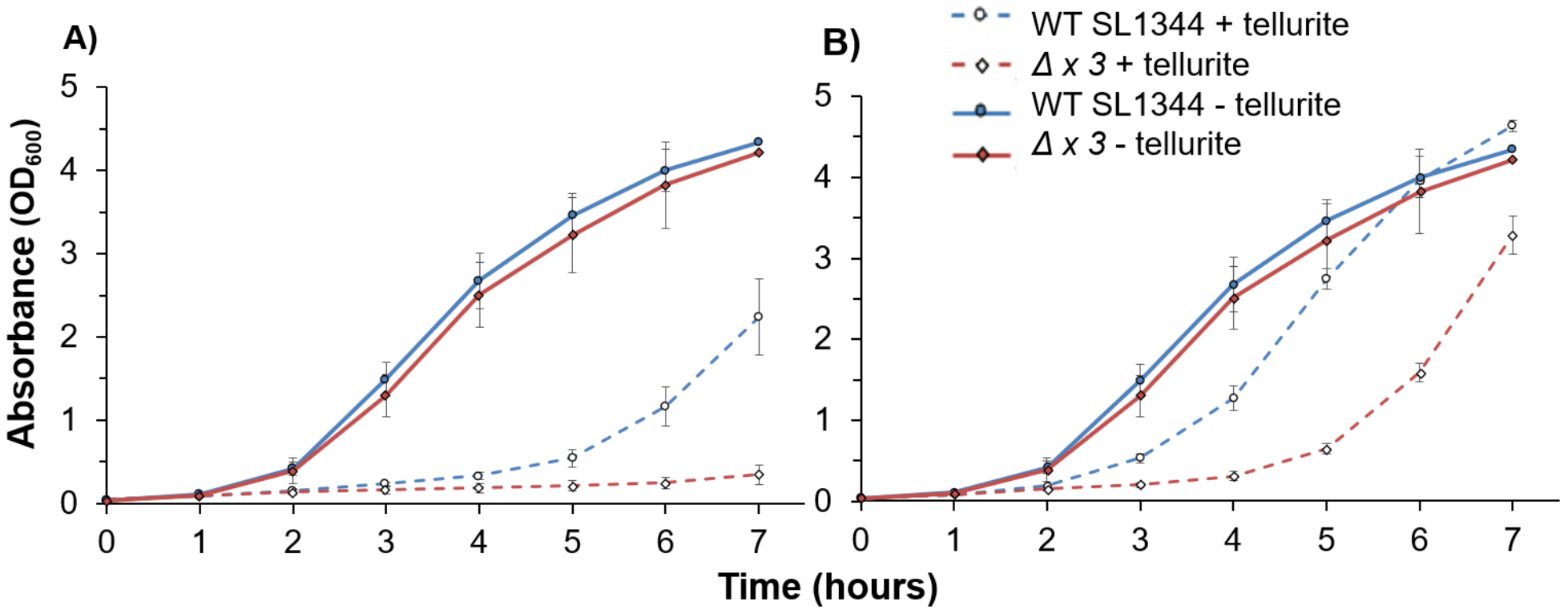


Figure 11. Treatment with 0.2 μM K_2TeO_3 severely attenuates the triple deletion strain with minimal impact on WT SL1344.
Strains (WT: circles, *ΔyeaR ΔSTM1808 ΔtehB*: diamonds) were grown in 50 mL LB and optical density readings were taken at 600 nm every hour for the duration of 7 hours with (dashed line) and without (solid line) the addition of 0.5 μM K_2TeO_3 (A) and 0.2 μM K_2TeO_3 (B). Data is composed of three technical repeats and standard error is plotted for each strain.

The impact of K_2TeO_3 is more prominent in true aerobic conditions and are highlighted in Table 3. As seen below, growth rates of strains without the presence of K_2TeO_3 are not significantly different from one another. However, at 0.2 μM WT growth is decreased by 20%, and this is much larger for the triple mutant (54%). This trend is seen at 0.5 μM giving WT a 71% decrease and the triple suffers ~96% reduction in growth rate.

Table 3. Growth rates of strains when grown aerobically in the presence of 0, 0.2 μM and 0.5 μM tellurite.
Statistically analysed by strain and then concentration using ANOVA single factor with replication. Significance is $p<0.05$, ** denotes $p<0.01$ in comparison to WT in post hoc Tukey's Test of multiple comparisons. Data is composed of the mean growth rates of three technical repeats.

Strain	**Growth rate (μ) at concentration (μM):**			**Statistical significance between concentrations of the same strain**
	0	**0.2**	**0.5**	
WT SL1344	0.913	0.731 ns	0.262 **	$p<0.001$, $F_2=48.53$
ΔyeaR ΔSTM1808 ΔtehB	0.837 ns	0.386 ** **	0.037 ** ***	$p<0.001$, $F_2=53.1$
Statistical significance between strains of the same concentration	$p=0.59$, $F_1=0.559$	$p=0.002$, $F_1=19.34$	$p=0.003$, $F_1=17.38$	-

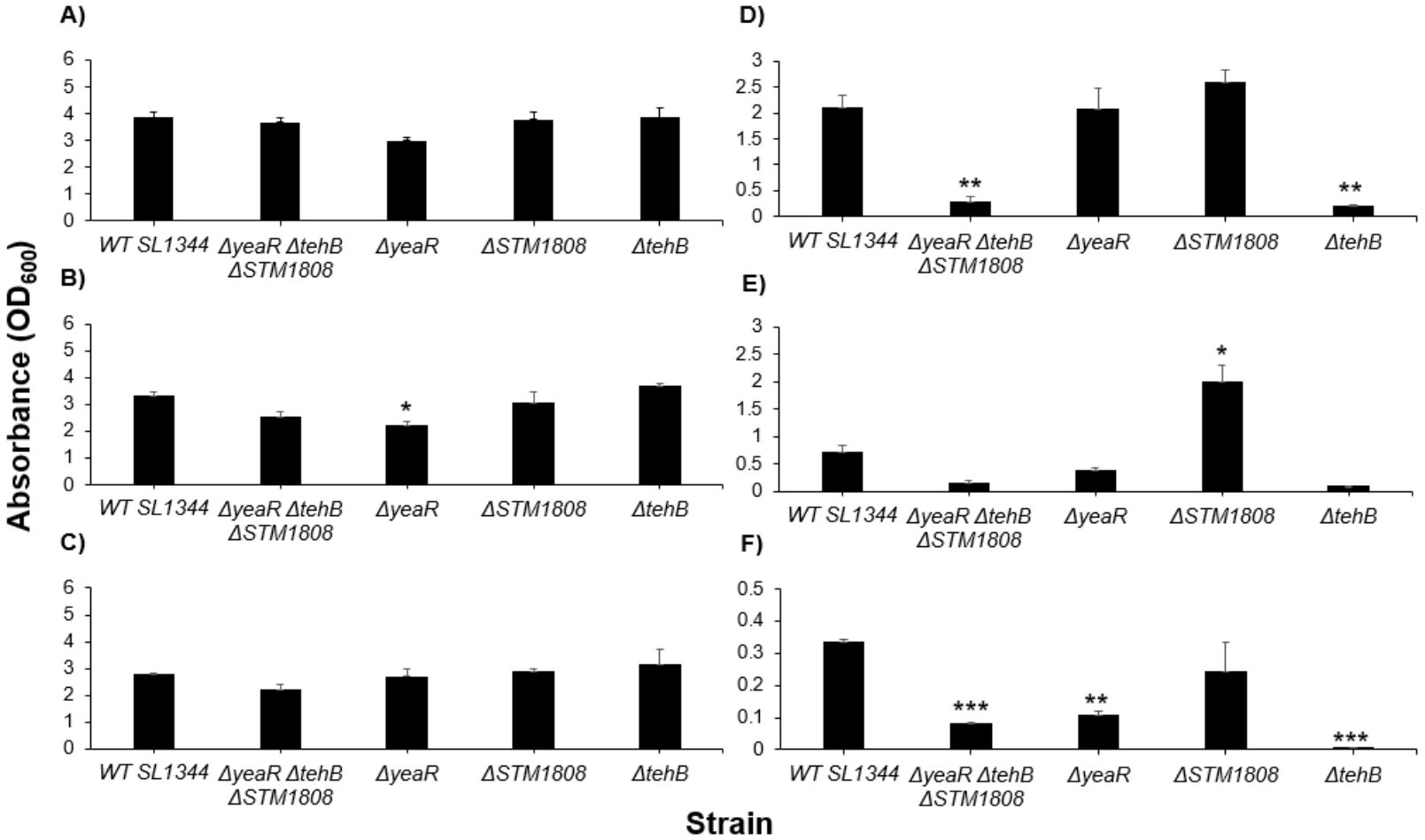


Figure 12. A higher resistance to tellurite is observed in aerobically grown liquid cultures.
Strains were grown for a period of 24 hours with no tellurite (A), 0.2 μM (B), 0.5 μM (C), 1 μM (D), 2 μM (E) and 5 μM (F). Data is composed of three technical repeats, standard error has been plotted and statistical analysis conducted using ANOVA single factor with replication and post hoc Tukey's Test with multiple comparisons. Asterisk (*) indicates significance: * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

3.3.3. TehB is more important for tellurite resistance despite functional redundancy between *ΔSTM1808*, *ΔyeaR* and *ΔtehB*

Minimum inhibitory concentration assays were utilised to understand the endpoint optical density readings after 24 hours. Various concentrations were trialled initially ranging from 0.1-10 μM. These experiments were conducted aerobically only.

There is a functional redundancy between YeaR, STM1808 and TehB proteins whereby the single deletion strains exhibit compensation. Due to the increased OD values of STM1808 in D, E and F it could be said that these are not representative of what should be seen. It could also indicate that STM1808 is not as imperative for tellurite resistance aerobically. Similarly, strains were grown overnight and plated on increasing concentrations of tellurite and Cfu/mL values were gained. This was done to support previous data and Cfu/mL values can be conceived as more accurate representation of growth compared to optical density readings.

Survival of *ΔtehB* is most impacted by tellurite addition and this deletion contributes most to the phenotype seen in the triple deletion strain compared to WT, especially in the 1-5 μM concentrations of K_2TeO_3. This suggests that TehB is the most important protein for eliciting tellurite resistance in WT *S.* Typhimurium SL1344 during aerobic exposure to tellurite. The *ΔSTM1808* and *ΔyeaR* mutants were still impacted by the addition of tellurite to the LB agar causing a significant reduction in survival compared to WT.

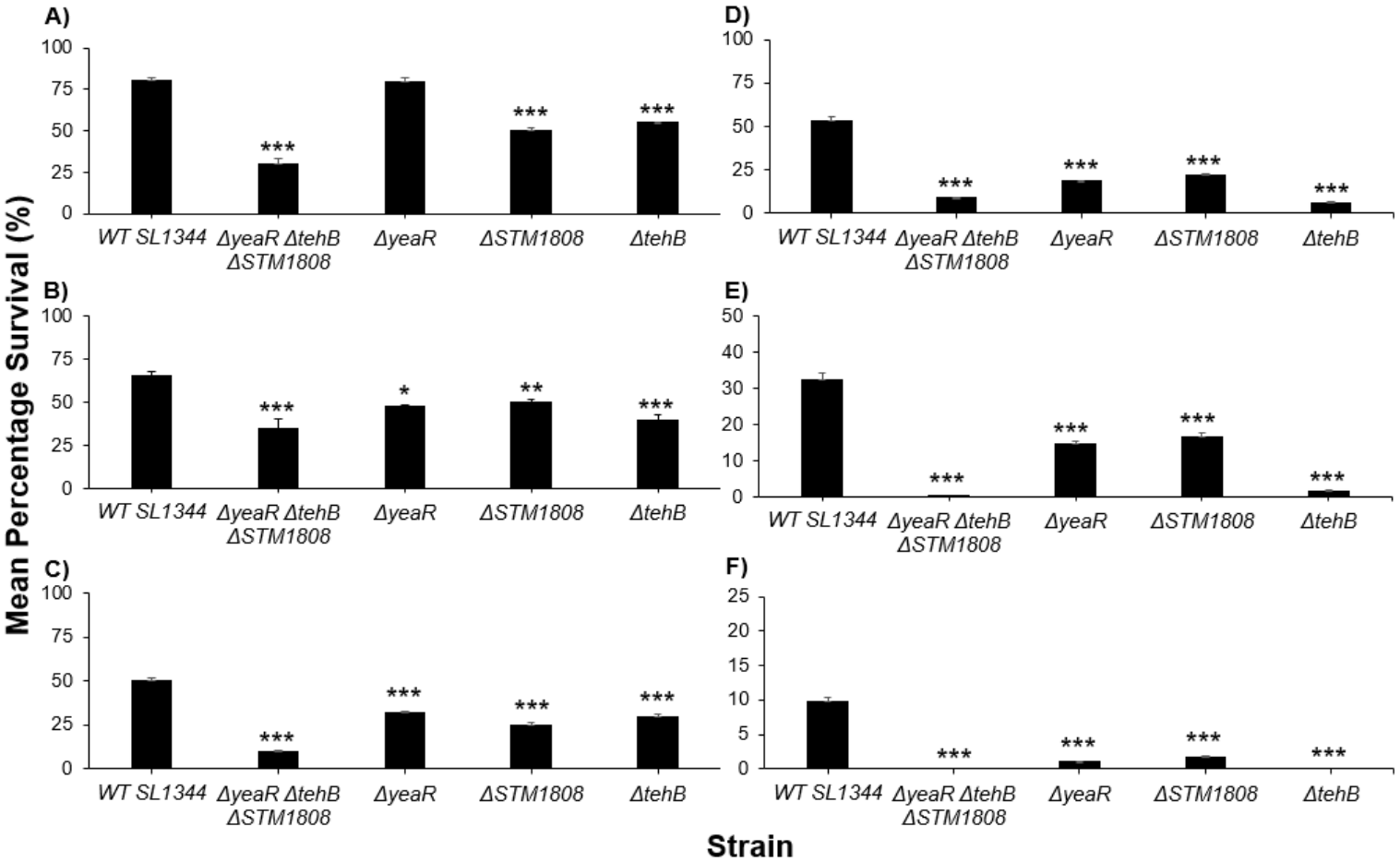


Figure 13. TehB plays a key role in aerobic survival during tellurite exposure.
Percentage survival from tellurite was calculated using Cfu/mL values on control plates (no K_2TeO_3) with Cfu/mL values containing 0.1 μM (A), 0.2 μM (B), 0.5 μM (C), 1 μM (D), 2 μM (E), 5 μM (F) K_2TeO_3. Data is composed of three biological repeats and standard error is displayed on bars. Statistical analysis was conducted using ANOVA single factor with replication and post hoc Tukey's Test for multiple comparisons to compare deletion strains to WT. Asterisk (*) indicates significance: * p<0.05, ** p<0.01 and *** p<0.001.

3.3.4. The addition of nitric oxide is detrimental to growth of mutants lacking in tellurite resistance proteins

Growth curves in aerobic and anaerobic conditions with the NO donor Deta NONOate were conducted with *STM1808*, *tehB* and *yeaR* deletion mutants. This is because these genes were previously shown to be upregulated post NO exposure in SL1344. The addition of 5 mM Deta NONOate was chosen to mimic NO exposure that *Salmonella* encounter during infection. This NO donor is known to spontaneously release 8 μM NO in LB which becomes halved after 24 hours treatment (Henard and Vazquez-Torres, 2012).

The addition of 5 mM deta NONOate results in decreased growth represented by lower OD readings. In WT, 30% growth is lost aerobically and 17% anaerobically. Whereas in the triple mutant growth is reduced 33% aerobically and this is much greater anaerobically being 50% loss in growth rate compared to growth without NO.

The single mutants were investigated in the response to NO due to lack of growth of the triple mutant to investigate if one of the proteins had a distinct phenotype suggesting a higher importance in NO detoxification.

Notably the NO phenotype of STM1808 is prominent both aerobically at 64% reduction and anaerobically at 62% reduction. YeaR and TehB appear to be less crucial aerobically as they only suffer 28% and 26% growth loss, respectively. However, they are more greatly impacted anaerobically resulting in ~50% reduction in growth rates for both strains.

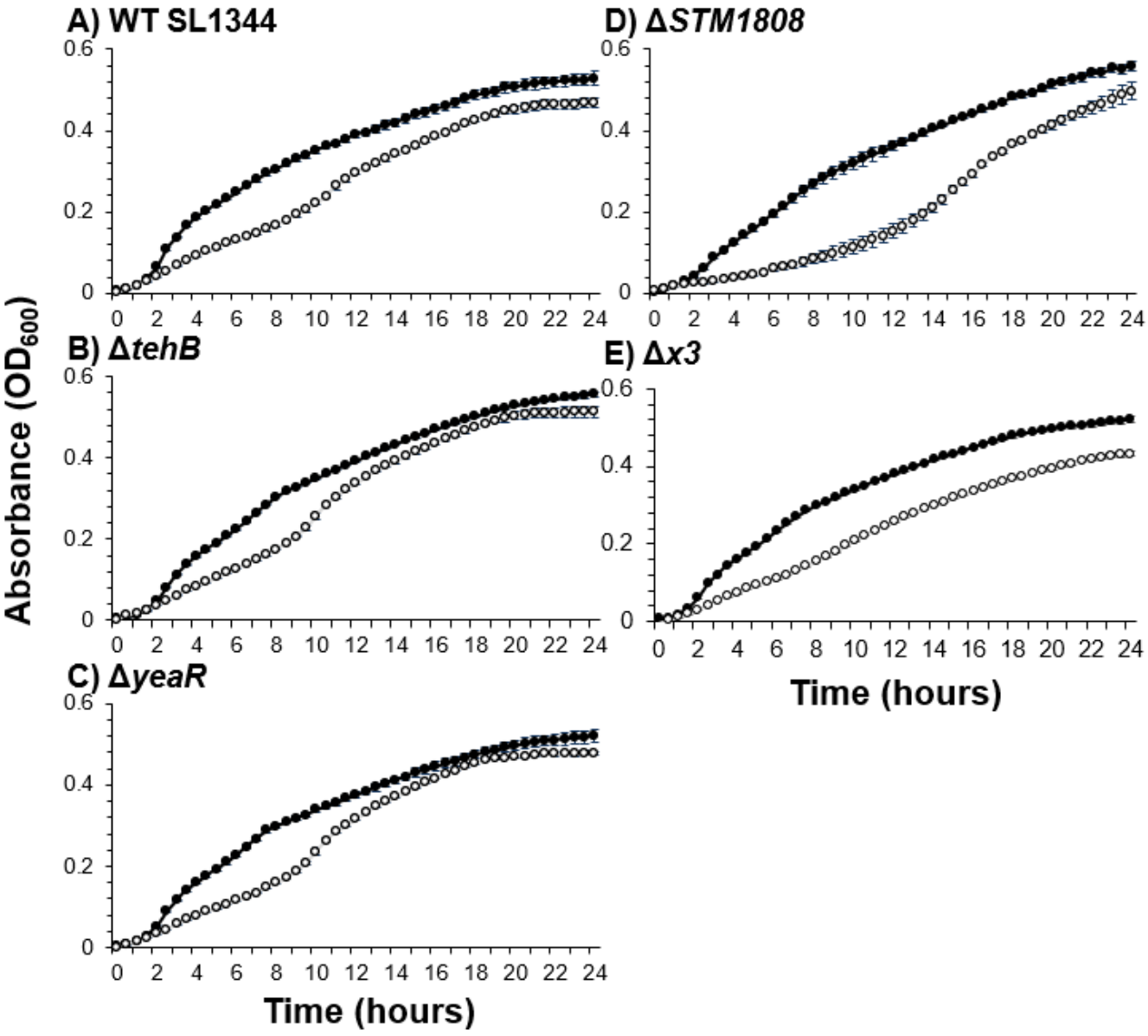


Figure 14. STM1808 is important during aerobic NO exposure.
Strains were grown in 1 mL of LB with (white circles) and without 5 mM deta NONOate (black circles) in a 24-well plate and automated optical density readings at 600 nm were taken every 30 minutes over a period of 24 hours in aerobic conditions. A) WT SL1344, B) *ΔtehB*, C) *ΔyeaR*, D) *ΔSTM1808*, E) Δ*x3*. Data is composed on three biological repeats and standard error is plotted on bars.

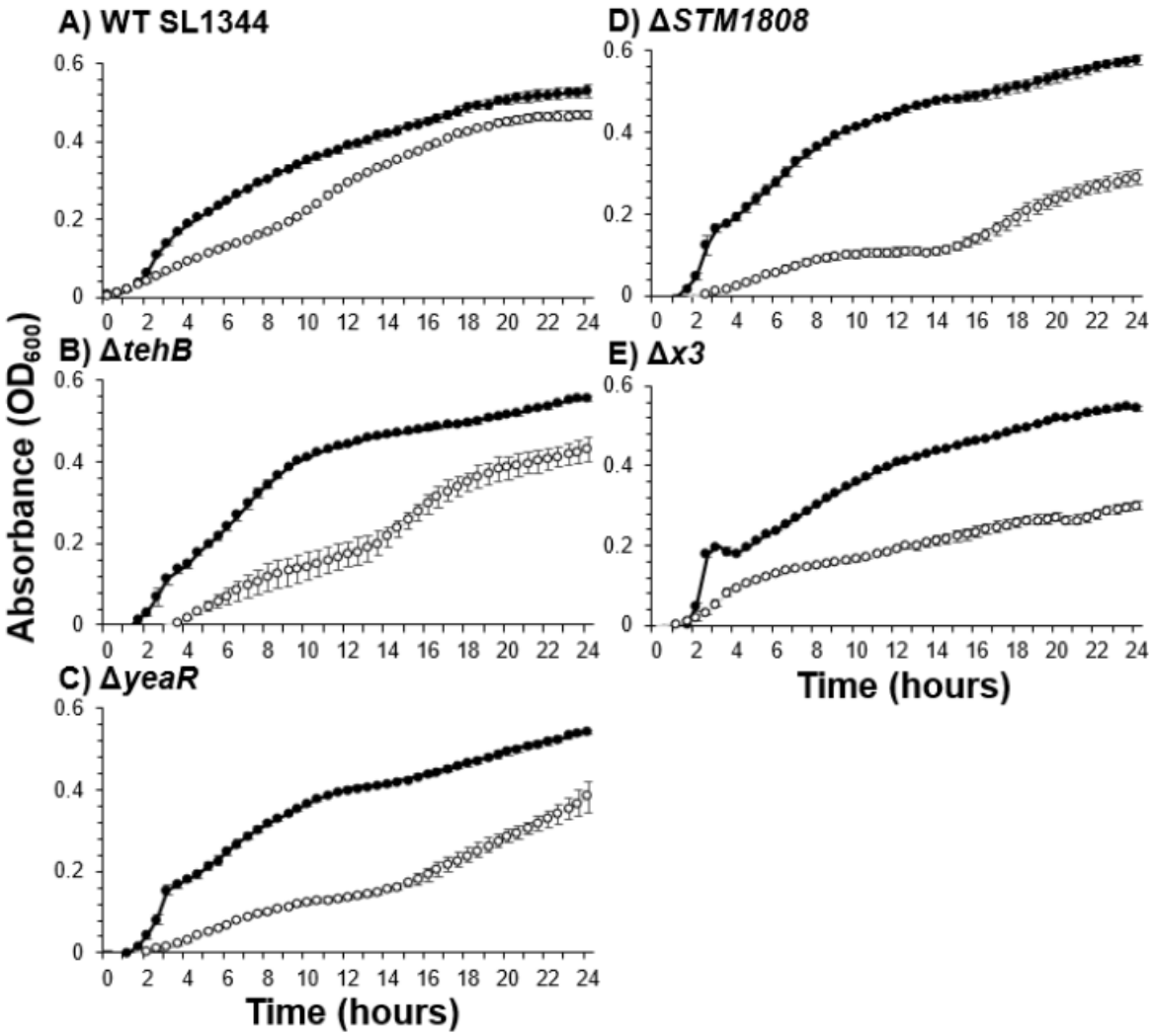


Figure 15. Tellurite resistance proteins are equally important in anaerobic NO protection. Strains were grown in 1 mL of LB with (white circles) and without 5 mM deta NONOate (black circles) in a 24-well plate and automated optical density readings at 600 nm were taken every 30 minutes over a period of 24 hours in anaerobic conditions. A) WT SL1344, B) *ΔtehB*, C) *ΔyeaR*, D) *ΔSTM1808* and E) Δ*x3*. Data is composed on three biological repeats and standard error is plotted on bars

3.3.5. Tellurite resistance proteins are conserved among some *Salmonella* serovars

A bioinformatic approach was used to see if these putative tellurite resistance proteins are found in host specific serovars. It is known that many other gram-negative bacteria possess homologs of these proteins which contain certain differences. Moreover, uncovering their dominance in *Salmonella* serovars will support the suggestion that these proteins have importance in other metabolic mechanisms.

Table 4. Conservation of STM1808 in *Salmonella enterica* serovars and *S. bongori.*

Serovar/species	Conserved?	Percent amino acid identity to Typhimurium (%)
Typhi	Yes	99.1
Dublin	Yes	100
Bongori	Yes	95.6

As seen from Table 4, a copy of STM1808 is found in *S*. Typhi, *S.* Dublin and *S. bongori* but some of these contain different amino acids which could be a result of mutations or be a natural process of evolution. From the comparison it was observed that all these proteins contained 119 amino acids and the DUF1971 domain remained unchanged. Typhi contained a F_{19}>T substitution and surprisingly Dublin had no differences compared to Typhimurium. In *bongori* the differences are more pronounced with several substitutions (E_{22}>D, T_{58}>A, K_{65}>T, E_{105}>D and E_{106}>Q).

Table 5. Conservation of YeaR in *Salmonella enterica* serovars and *S. bongori.*

Serovar/species	Conserved?	Percent amino acid identity to Typhimurium (%)
Typhi	Yes	99.2
Dublin	Yes	99.2
Bongori	Yes	89.9

These variations of YeaR were all 119 amino acids in length, and all contained at least one substitution from YeaR found in *S.* Typhimurium which explains the percent identity seen in Table 5. The DUF1971 domain was also conserved among the serovars, and species

tested. The substitution seen in Typhi, and Dublin is exactly the same being D_{37}>G, whereas in *S. bongori* there are several changes such as R_2>L, H_8>Y, E_{20}>A, T_{21}>S, D_{37}>G, C_{60}>S, S_{61}>P, I_{66}>V, V_{68}>I, A_{109}>T, T_{110}>R and A_{117}>T.

Table 6. Conservation of TehB in *Salmonella enterica* serovars and *S. bongori.*

Serovar/species	Conserved?	Percent amino acid identity to Typhimurium (%)
Typhi	Yes	99.5
Dublin	Yes	100
Bongori	Yes	93.9

Similar levels of identity were observed across the TehB and STM1808 variations. All the TehB proteins were 198 amino acids in length which is similarly observed in *S.* Typhimurium. No substitutions were found in Dublin and only one was found in Typhi which was D_{140}>G (Table 6). In *S. bongori* the differences are obviously more prominent as the percent identity was 93.9% (table 6). Amino acid substitutions included D_{21}>E, A_{63}>V, A_{74}>T, D_{83}>S, L_{84}>I, T_{89}>A, T_{91}>S, I_{167}>L, P_{138}>E, T_{197}>P and A_{198}>V.

3.4. Discussion

The toxicity of tellurite is known to involve the generation of ROS. Mechanisms which have been discussed in the literature are specific to bacteria that occupy particular niches (Taylor, 1999, Maltman and Yurkov, 2019). Although some commonalities between tellurite resistance have been observed for example reductases employed that are dependent on NAD(P)H and cysteine residues (Arenas-Salinas et al., 2016, Calderón et al., 2006). Speculation remains about the physiological relevance of tellurite resistance genes particularly for organisms that are not commonly exposed to tellurite. This is true for TehB, YeaR and even *Salmonella* specific STM1808 tellurite resistance proteins. Previous work by Arkenberg (2013) highlighted the importance of *STM1808*, *yeaR* and *tehB* which were all significantly upregulated in SL1344 post exposure to NO. It was also proven that *STM1808* and *yeaR* became significantly upregulated following tellurite exposure. Empirical evidence was obtained in that study that tellurite toxicity was reliant on ROS because strains supplemented with a ROS scavenger allowed recovery of growth phenotypes during tellurite exposure. Furthermore, Johnston (2017) proved two histidine (H32 and H82) residues which were found in the DUF1971 domain in SMT1808 and YeaR were crucial for NO protection which supported Karlinsey et al. (2012). However, these residues were not important for tellurite resistance. This chapter aimed to

uncover the importance of STM1808, TehB and YeaR in tellurite resistance and NO detoxification in aerobic and anaerobic conditions. This was investigated by assessing the phenotypes of various deletion mutants lacking in *STM1808*, *yeaR* and *tehB* in the presence of low concentrations of tellurite and 5 mM deta NONOate.

Firstly, obtaining proof that strains grew similarly to WT SL1344 allowed further phenotypic experiments to take place with confidence that growth defects would only be the result of the stress elicited to *Salmonella* Typhimurium. As a facultative anaerobe, *Salmonella* should experience no difficulty respiring with oxygen or an alternative electron acceptor which is proven here. However, creating a true anaerobic environment can be difficult in the plate reader and may result in strains growing aerobically initially until oxygen has been entirely used and this can cause small discrepancies in the data. The overlapping error bars supported that no significant growth differences were witnessed among strains.

TehB has been widely investigated in the literature, particularly in *E. coli*. In contrast to *E. coli*, TehB in *Salmonella* lacks in a *S*-adenosyl-L-methionine (SAM) domain which is argued to be important for the methyltransferase function (Cournoyer et al., 1998). However, the biochemical function of TehB in *Salmonella* has not yet been elucidated, but it cannot be ruled out that there are other cofactors present which allow the enzyme to function for tellurite resistance. Domains like this do not exist in YeaR or STM1808 which point to these proteins having distinct mechanisms for tellurite resistance. In this study it was evident that STM1808, YeaR and TehB had importance in tellurite resistance both aerobically and anaerobically due to the decreased growth phenotypes seen. There appears to also be a functional overlap in these proteins provide because *ΔSTM1808* and *ΔyeaR* exhibit higher growth than *ΔtehB* during tellurite exposure which could be explained by the upregulation of other active systems resulting in compensation.

It became apparent that *ΔtehB* followed the trend of exhibiting lower growth than other single mutants *ΔSTM1808* and *ΔyeaR* which would suggest that TehB has a more prominent role in tellurite protection during aerobic conditions. This supports work done by Arkenberg (2013) who suggested that the deletion of *tehB* was the greatest contribution to attenuation observed in *ΔyeaR ΔSTM1808 ΔtehB*; however, the additional mutations further negatively impacts growth. It is known that tellurite is a cytotoxic chalcogenide much more so than related compounds derived from Selenium (Turner et al., 2001). Meaning there is no surprise that attenuation in growth is observed in concentrations as low as 0.1 μM particularly for deletion mutants that lack in tellurite resistance proteins.

The decrease in growth seen in WT is higher than the triple mutant because it still has functional NO detoxification systems. Interestingly, the phenotype seen in the triple mutant

during anaerobic and aerobic NO exposure is mostly due to the deletion of *STM1808*. This is because growth of *ΔSTM1808* during exposure to NO in aerobic and anaerobic conditions was extremely diminished. This reassuringly confirms the importance that STM1808 has in response to NO (Karlinsey et al., 2012). During anaerobic conditions, *ΔyeaR*, *ΔtehB* and *ΔSTM1808* experience similar attenuation which may highlight functional overlap among these proteins in NO protection. Whereas, in aerobic conditions *ΔyeaR* and *ΔtehB* could be said to have a lesser role in NO detoxification because growth with deta NONOate was similar to WT.

Salmonella reliance on detoxifying ROS and RNS to enable macrophage survival gives these tellurite resistance genes physiological relevance. The evolutionary development of enzymes capable of detoxifying hydroxyl radicals is valuable regardless of enabling tellurite resistance. The link that is apparent between NO and tellurite either directly or indirectly result in ROS generation which ultimately require detoxification, and this could be done using related mechanisms or employ the use of similar enzymes. To investigate further, tellurite resistant mutants will be generated and analysed for genetic and phenotypic differences. It would be worth examining if the strains generated have altered ROS or RNS profiles which have become apparent due to increased tellurite resistance (see chapter 4).

Despite diverging from *E. coli* millions of years ago, *Salmonella* remains to have numerous areas of synteny which is why research into genes can overlap between the enteric pathogens. It is a fact that *Salmonella* have diverged using horizontal gene transfer resulting in genetic drift. Acquiring such genes allows for full virulence even specialisation of *Salmonella* strains and some of these characteristics were developed after diverging from the common ancestor between *S. enterica* and *S. bongori*. It was reported by Fookes et al. (2011) that *S. bongori* possesses a limited, ancestral set of virulence genes, further lacking in metabolic pathways that distinguish *S. enterica*. For example, the absence of SPI-2 may contribute to the lack of infection seen in warm-blooded hosts. It is known that SPI-1 is one of the most highly conserved gene clusters which is seen across all serovars. Interestingly, *S.* Dublin retained the Typhimurium virulence plasmid (pSLT) that contains the *spv* cluster which is required for generating a systemic infection in mice (Chan et al., 2003). Such plasmid is not present in *S.* Typhi or *S. bongori.* As previously mentioned the *avrA* gene is only present in human specific serovars excluding typhoidal serovars, but the gene has been identified in *S. bongori* (Giacomodonato et al., 2014).

Assessing the prevalence of tellurite resistance proteins can allow the understanding of their importance in *Salmonella* and the likelihood of having dual functionality in other metabolic processes that may aid virulence. Interestingly, STM1808, YeaR and TehB proteins were

found in one form or another in *S.* Typhi, *S*. Dublin and *S. bongori*. It was observed that STM1808 contained higher percent identities across homologs identified in the serovars investigated. This being said, YeaR and TehB were still conserved among the groups, but a larger number of substitutions were found. Remarkably, any mutation that may have resulted in amino acid substitutions did not result in premature stop codons so it could be presumed that none of the homologs identified were truncated, but to assess protein functionality attention should be directed to the amino acids themselves. In fact, the genetic redundancy present in protein coding can allow for amino acid substitutions to occur and these are not typically disastrous if they have similar properties, unless the amino acid has a key role in protein structure or function.

DUF1971 containing homologs of Typhimurium STM1808 and YeaR proteins still displayed conserved histidine residues (H32 and H82) which have been previously reported to be crucial in how the proteins function (Karlinsey et al., 2012). A third of substitutions in STM1808 homologs had no impact on the amino acid properties out of the 6 differences observed. Meaning it is unlikely the changes would have a detrimental effect on the protein function. But one change from phenylalanine to threonine which would have the addition of a reactive hydroxyl group and methyl group to the protein. The other substitution was threonine into alanine which are both small amino acids. There were conversions from a negative (glutamic acid) and positive (lysine) amino acids to polar neutral amino acids (glutamine and threonine, respectively) which occurred in the other third of substitutions.

In YeaR of the 13 substitutions seen, 23% would have resulted in the same amino acid properties. However, 15% have a hydrophobic amino acid changed into a polar amino acid and the same percentage is seen for the conversion of a positively charged amino acid to ones with a hydrophobic side chain. Less common is the substitution of a hydrophobic amino acid from a negatively charged amino acid (8%). The transformation to glycine from aspartic acid allows the introduction of a unique structure which is able to bind to phosphate and is a key property in protein kinases. The introduction of proline from serine is likely to have impacted the secondary structure of the protein. This is because proline usually occurs where the polypeptide changes direction or kinks to allow for α-helices and β-pleated sheets. This small amino acid can be substituted with other small amino acids, but because of the unique structure of the side chain connecting to the protein backbone twice, it doesn't substitute well (Betts and Russell, 2003). The loss of cysteine could be detrimental to YeaR as this amino acid contains a reactive sulfhydryl group that allows the formation of disulphide bridges in the permanent primary protein structure. Cysteines are frequently present in catalytic, regulatory or cofactor regions in proteins. For example, in thiol oxidoreductases these conserved residues participate in reduction, oxidation or the isomerization of disulphide bonds. Other

functions for this amino acid include coordinating metal binding or even becoming sites for posttranslational modifications (reviewed in Fomenko et al. (2008)). The alternative gain of serine can further alter structure as this amino acid is able to make hydrogen bonds with many substrates which is why they are commonly present in protein functional centres (Betts and Russell, 2003).

In the case of TehB, 50% of the substitutions did not change the original amino acid characteristic that being negative, polar or hydrophobic. There was one case of threonine, a polar amino acid, substitution to alanine which is hydrophobic and vice versa which is quite common in proteins (Betts and Russell, 2003). Similarly, there was one substitution that resulted in a polar amino acid which was negatively charged in Typhimurium. There was also two cases that involved special amino acids where aspartic acid was converted to glycine and where proline was converted into glutamic acid. As previously mentioned, TehB contains three cysteine residues which have a crucial role in tellurite resistance (Dyllick-Brenzinger et al., 2000). None of the cysteine's were substituted in the homologs of TehB which is supporting evidence that these proteins are functional for the purpose of tellurite resistance. At the C-terminus of TehB in *S. bongori* two substitutions are seen being threonine to proline which could have effects on protein structure and alanine to valine which would unlikely impact the protein.

Future studies should focus on the purification of STM1808, YeaR and TehB to solve the protein structure and to understand the biochemical processes used for tellurite resistance. This will allow us to understand how the amino acid residues interact with one another and if the substitution of certain amino acids could be detrimental to protein structure or function. It would also be useful to conduct experiments during nitrosative stress. To definitively know that these proteins have a role in both tellurite resistance and or NO detoxification, overexpression mutants of individual proteins could be made. These would theoretically display similar phenotypes to *ΔnsrR*, but it may give more information on how each protein contributes to tellurite resistance and nitrosative stress responses. An obvious avenue that requires further exploration is studying the expression of these genes particularly in other *Salmonella enterica* serovars and *S. bongori* to assess if expression patterns are similar to Typhimurium. This would not only confirm bioinformatic investigation into their conservation and allow the discovery of mutation consequences in other serovars, but it would also provide insight if they possess the capacity for tellurite and NO resistance.

Chapter 4: Using directed evolution to develop tellurite resistance in *Salmonella*

4.1. Introduction

As previously stated, tellurium is a rare element that has four stable oxidation states including the toxic form, tellurite. Tellurite is not prominent in the environment, but it has been found in high concentrations in hydrothermal vents, thermal springs, hypersaline environments, and mine tailings. Areas where *Salmonella* would not usually occupy during their life cycle. Microorganisms that are able to inhabit these locations have the ability to tolerate high concentrations of tellurite possibly due to selective pressures to evolve resistance (Maltman and Yurkov, 2019). The ability for these extremophilic bacteria to survive is dependent on reducing the oxyanion to elemental tellurium which is considered non-toxic. As discussed previously, these bacteria use widely different mechanisms to reduce tellurite to form tellurium crystallites within the cells. These are known to accumulate in the periplasm of *Rhodobacter spaeroides* or in the inner plasma membrane of *E. coli* (Trutko et al., 2000). Tellurite is known to gain entry to cells using the ActP and PitA transporters in *E. coli* (Elías et al., 2015), but little is known about tellurite and tellurium transport in *Salmonella*. Evolutionarily, there would be no reason for the transport of such toxic elements unless they resembled useful homologs.

Antibiotics are heavily relied upon to treat severe *Salmonella* infections despite increasing AMR. These drugs can vary in targets which allow them to be useful against many pathogens (broad-spectrum) or specific microorganisms (narrow-spectrum). Gentamicin is a broad-spectrum antibiotic which interferes with protein synthesis. As an aminoglycoside, gentamicin, can bind to the bacterial surface electrostatically which as a result displaces divalent cations, increasing the permeability of the membrane and allowing entry to the periplasm. To subsequently enter the cell, the antibiotic requires energy dependent transport which occurs through porins. Resistance is known to occur when there are defects in porins such as OmpC and OmpF in *E. coli* (Serio et al., 2018). Ceftriaxone is another broad-spectrum antibiotic which works similarly to other β-lactam antibiotics by inhibiting peptidoglycan formation. Bacteria can become resistant by overproducing extended spectrum β-lactamases, also known as cephalosporinases, which reside on mobile genetic elements such as transposons or plasmids (Al kraiem et al., 2018). Polymyxins are small cationic peptides which electrostatically interact with the LPS and bind Lipid A in the outer membrane increasing permeability in the cell membrane which ultimately leads to death. Polymyxins can additionally result in the production of hydroxyl radicals which can be lethal (Sampson et al., 2012). Resistance has been recorded in *Salmonella* including mutations present in *phoPQ* and *pmrCAB* which result in changes to the LPS to diminish the binding abilities of the antibiotic (Li et al., 2019).

ROS detoxification mechanisms have been linked with tellurite resistance such as *sodAB* superoxide dismutase activity and the *soxS* oxidative stress response as the deletion leads to tellurite sensitivity (Pérez et al., 2007). Certain classes of antibiotics are known to be bactericidal through producing ROS including β-lactams, quinolones, aminoglycosides, glycopeptides and polymyxins (reviewed in Zhao and Drlica (2014)). RNS have also been linked with antibiotics in the literature and in a study conducted by Ribeiro et al. (2021) the pre-treatment of *E. coli* with NO was found to give the bacterium a higher tolerance to gentamicin. Contrastingly, supplementation of alanine alongside gentamicin enhanced the effect of antibiotic facilitated killing by inhibiting arginine mediated production of NO (Kuang et al., 2021). However, earlier investigations by Coban and Durupinar (2003) on *Salmonella* clinical isolates showed the combination of Deta-NO with ofloxacin, ciprofloxacin and pefloxacin did not have a synergistic effect. Although, they did identify an antagonistic effect on the antibiotics when treated with NO for some strains which contained mutations in *soxRS* and *acr*.

Early research articles suggest the transference of almost 80% tellurite resistance occurred in enterobacteria via plasmids that also contained antibiotic resistance which were sampled from sewage systems and rivers (Smith et al., 1978). These findings were supported by Makino et al. (1981) where multiple resistance characteristics where identified and ~75% appeared to be easily transmitted between species. Similarly, an investigation conducted in Chile on *S. panama* found that most clinical isolates were resistant to nitrofurans, streptomycin, spectinomycin, sulfonamides, tetracycline, mercuric and tellurite salts (Cordano and Virgilio, 1996). However, more recent publications suggest the resistance against antibiotics in clinically relevant pathogens have ‘traded off’ resistance to heavy metals. An article by Figueiredo et al. (2019) isolated *Salmonella* from food animals and food products. Following genotyping, they identified up to 12 different mobile AMR genes. Out of these studied, the organisms appeared to be more susceptible to zinc, copper, silver, arsenic and tellurite. They suggest that heavy metals enables the selection of AMR genes through the food chain, particularly for zoonotic pathogens like *Salmonella*.

Directed evolution in the context of protein engineering, refers to the use of natural selection pressures to steer proteins or nucleic acids to a defined goal (Arnold et al., 2001). Whereas, in the context in bacterial evolution it can mean the random or targeted development of mutations when subjected to a mutagen stimulus whereby adaptive resistance is generated (Vávrová et al., 2021). The development of point mutations can be developed easily in bacteria and these can be beneficial to the organism which would warrant retaining the mutation or it can be harmful and lead to damaging results if not repaired (Wrande et al., 2008). Mutations could be the resistance or susceptibility to antimicrobials, impacting virulence or metabolic

functions which could be advantageous or detrimental. The likelihood for beneficial mutations to be passed on to subsequent progeny is favoured particularly if it confers competitive advantages (Granato et al., 2019).

Research conducted by Morales et al. (2017) suggested that tellurite targets the haem biosynthetic pathway. Under aerobic conditions, protoporphyrin IX accumulates due to lack of Fe and as a result hydroxyl radicals and singlet oxygen are formed which can be tolerated by cells that are not undergoing other stresses. However, when subjected to tellurite, the intracellular iron levels are depleted, and diminished pools of thiols reduces production of hydroxyl radicals and singlet oxygen. This cascade overcomes defence systems and ultimately leads to cell death. Controversially, they found that hydrogen peroxide or superoxide did not play a key role, but hydroxyl radicals did participate in tellurite toxicity in *E. coli*.

In the paper by Morales et al. (2017) they developed a directed evolution experiment whereby they generated increased tellurite resistance in *E. coli* BW25113. The strain denoted EM2 was isolated after 26 days of passaging at increasing concentrations of tellurite. After generating EM2 they subjected the strain to sequencing where they identified mutations in *hemA*, a gene within the heme biosynthetic pathway. It appeared the mutations they generated became targets to investigate how tellurite elicits toxicity in *E. coli* cells (Morales et al., 2017). For this reason, their method was adapted in this chapter to generate mutations in *Salmonella* Typhimurium and other phenotypes for hydrogen peroxide and antibiotics were explored to understand the consequences of evolved tellurite resistance. Their study did not explore how these mutations impacted virulence of the strain, for example macrophage survival. They used a non-virulent laboratory strain of *E. coli* which is not typically used for macrophage studies. Macrophage infection and survival of tellurite resistant strains will be investigated in this chapter.

4.2. Chapter aims

The introduction has outlined the prominence of tellurite resistance in various organisms and how this resistance can be obtained within a laboratory *E. coli* strain; therefore, it was hypothesised that this can be repeated in *Salmonella* through adapting the methodology used by Morales et al. (2017). There is emphasis on how heavy metals such as tellurite can allow the selection of AMR genes and how this can be of clinical importance for zoonotic pathogens in the literature. Furthermore, the evolution of bacteria can occur rapidly, and it is not uncommon of mutations that provide beneficial phenotypes becoming adopted. Thus, consequences of resistance cannot necessarily be predicted due to the random nature of mutations that develop during passaging. However, logically the development of more efficient

enzymes that can combat stress elicited by ROS or RNS would be particularly favourable in a tellurite resistant strain and may impact the strains ability to withstand antibiotics that elicit ROS or H_2O_2 as their mode of antibacterial action. Therefore, it was hypothesised that induced tellurite resistance would allow resistance to other stressors such as NO, H_2O_2 and antibiotics indirectly. Moreover, possessing these characteristics would also benefit the tellurite resistant strain during infection and survival within macrophages.

This chapter aimed to:

1. Evolve tellurite resistance in WT SL1344 and *ΔyeaR ΔSTM1808 ΔtehB* strains by using an adapted directed evolution approach.
2. Phenotypically characterise alternative gain or loss of function mutations including resistance or sensitivity to Nitric oxide, hydrogen peroxide, antibiotics and survival in macrophages.
3. Identify changes in genotype between passaged tellurite resistant strains and non-passaged tellurite sensitive strains.

Aims were achieved by:

- Conducting pilot experiments by changing variables such as time passaged, concentrations of tellurite used and passage method until the right conditions were identified to conduct directed evolution of WT and *ΔyeaR ΔSTM1808 ΔtehB* strains.
- Phenotypic screening against NO, H_2O_2 and antibiotics (gentamicin, ceftriaxone and polymyxin B) in growth curves, percentage survival and disc diffusion assays.
- Assessing the impact of macrophage infection and survival of the tellurite resistant strain generated using the WT background and comparing to the parent strain.

4.3. Specific Methodology

4.3.1. Directed Evolution Pilot

Preliminary directed evolution experiments were conducted with wildtype SL1344 to test concentrations and to assess the number of passages required until the next concentration was introduced. Passage techniques were tested using method 1: inoculating fresh LB media with 1/1000 (v/v) dilution of the grown O/N and tellurite added in the right concentration, or method 2: cells were spun down in a microcentrifuge at maximum rpm for 60 seconds and pellets were resuspended in fresh LB media and the relevant concentration of tellurite was added. Both methods worked effectively and resulted in growth observed the following day but method 2 allowed all resistant cells to continue to grow and not a small sample. It was

deduced that this method would be best for introducing the cells to a higher concentration as it would maximise the chances of the cultures to grow.

4.3.2. Directed Evolution Final Experiment Design

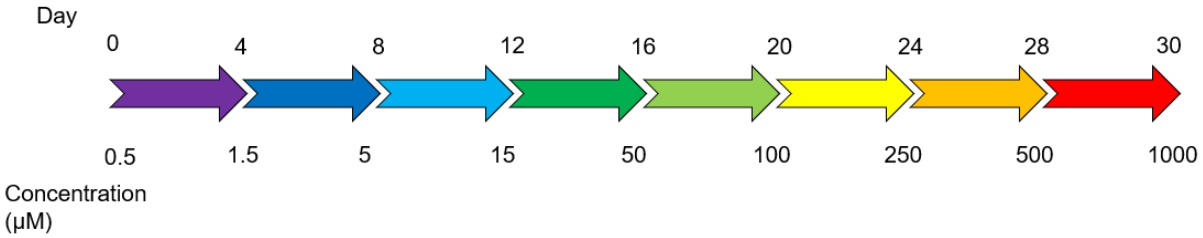


Figure 16. Directed evolution experimental design.
Day passaged (defined by 24 hours) and concentration of tellurite (μM) used is shown here. Inspired by Morales et al. (2017) and preliminary experiments conducted in wildtype SL1344.

Wildtype SL1344 and *ΔyeaR ΔSTM1808 ΔtehB* were grown with a starting concentration of 0.5 μM K_2TeO_3 in standard LB O/Ns in 37 °C incubation with shaking at 180 rpm. To passage cells method 1 was used generally but at key points of where the concentration increased seen in Figure 16, the cells were passaged using method 2 stated above. At the end of each concentration aliquots were taken from O/Ns to inoculate a 24-well plate to conduct 24-hour growth curves shown in 4.5. These results would display a control SL1344, control *ΔyeaR ΔSTM1808 ΔtehB* with and without tellurite and passaged SL1344 and passaged *ΔyeaR ΔSTM1808 ΔtehB* (noted TR accordingly) with and without tellurite. At concentrations 500 μM and 1 mM, the passaged cells were plated out appropriately on tellurite containing and absent plates to compare growth with non-passaged cells. Five single colonies of equal size and morphology were then patched onto a new 1 mM tellurite plate and two of the patches per strain were used to inoculate Microbank™ beads (ProLab Diagnostics) according to manufacturer's instructions and stored at -80 °C for long-term storage. Four tellurite resistant strains were made into stocks, two for each background. Strains (WT SL1344 and TR SL1344) were sent for whole genome sequencing using the manufacturer's (microbesNG) instructions.

4.4. Results

4.4.1. Resistance to tellurite can occur through gradual exposure to increasing concentrations

A directed evolution approach was used to generate enhanced tellurite resistance in WT SL1344. Concentrations of potassium tellurite were gradually increased according to the directed evolution methodology. Initially due to baseline resistance presence in SL1344, growth appeared to be similar at lower concentrations. However, the passaged WT (P SL1344) managed to exceed the baseline resistance present in WT which was seen up to 50 μM K_2TeO_3. Positive controls of each strain were still grown to ensure that any lack of growth was caused by the addition of tellurite. Initially there was no difference in the two strains, but as time passes with more mutations accumulated the passaged strain experiences slower growth without the tellurite at much higher concentrations.

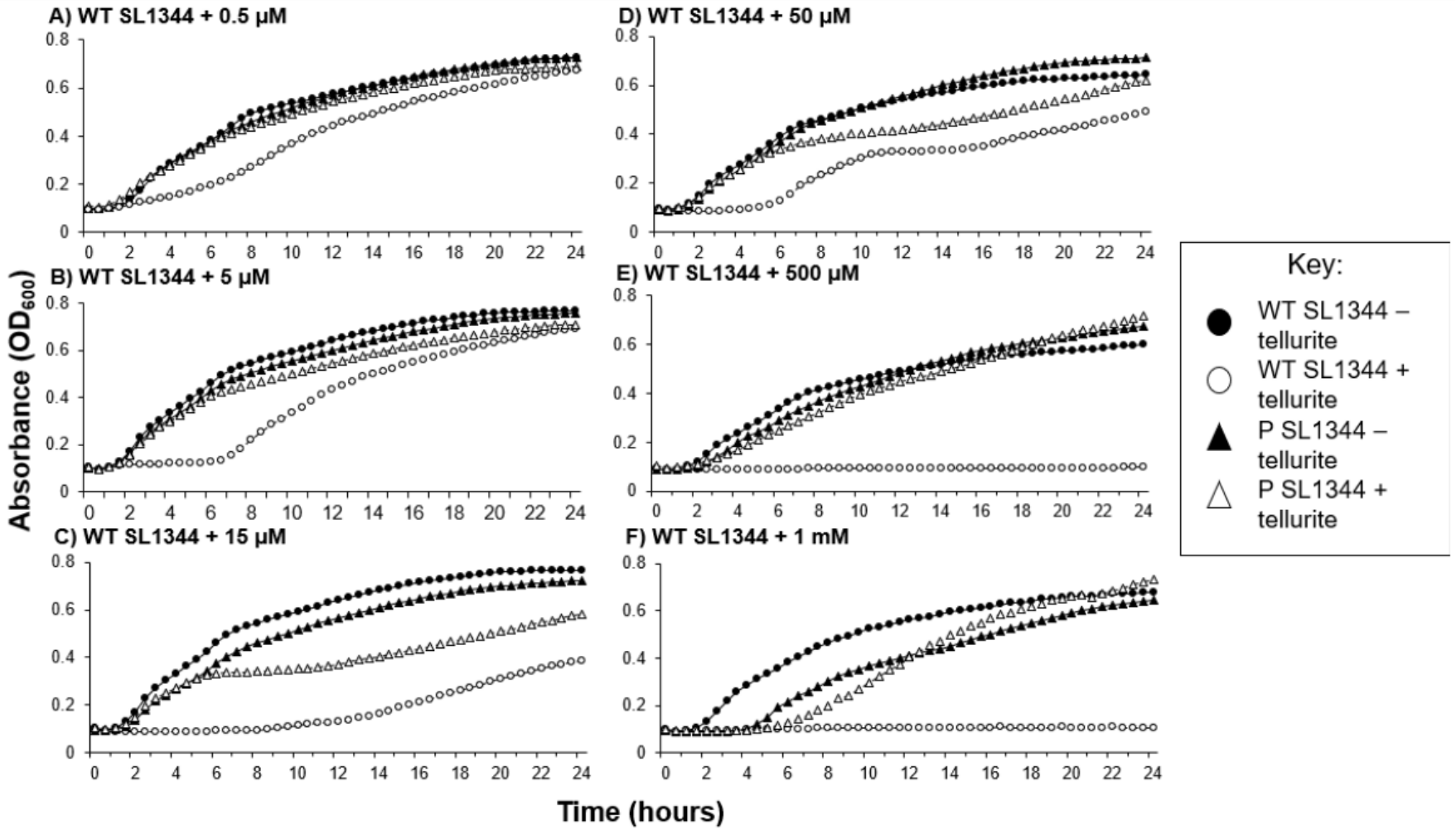


Figure 17. Generation of a tellurite resistant strain using WT SL1344 in a directed evolution approach by passaging over 30 days in various concentrations of tellurite.
A) 0.5 µM, B) 5 µM, C) 15 µM, D) 50 µM, E) 500, F) 1 mM. Growth curves were conducted after completing several passages in select concentration (over 3-4 days) and were collected in 1 mL LB 24-well plates and optical density at 600 nm measured over 30-minute intervals over 24 hours in a SpectraMax iD5 plate reader. WT SL1344 (circles) was grown alongside the passaged WT (triangles) as a control.

Additionally, tellurite resistance was generated in the *ΔyeaR ΔSTM1808 ΔtehB* strain by gradual exposure to higher concentrations to potassium tellurite in a similar way to WT. This experiment involved the addition of kanamycin as this antibiotic was required to ensure the deletion of the aforementioned tellurite resistance genes. This meant that any tellurite resistance observed in this strain was not due to reversion of previous mutations to tellurite resistance genes.

Lacking in the tellurite resistance proteins STM1808, YeaR and TehB gives a clear attenuated growth phenotype in the non-passaged triple mutant. Noticeably, the fitness of the passaged strain is decreased when not grown in the presence of tellurite as it does not grow similarly to the non-passaged triple mutant without tellurite. These results prove that a low level of tellurite resistance can be developed in the triple mutant which consequently limits the strains ability to grow without tellurite. This data suggests that there are other mechanisms responsible for tellurite resistance in *Salmonella*. Due to the lower resistance profile in *ΔyeaR ΔSTM1808 ΔtehB*, tellurite resistance was consequently more difficult to obtain than in the WT background.

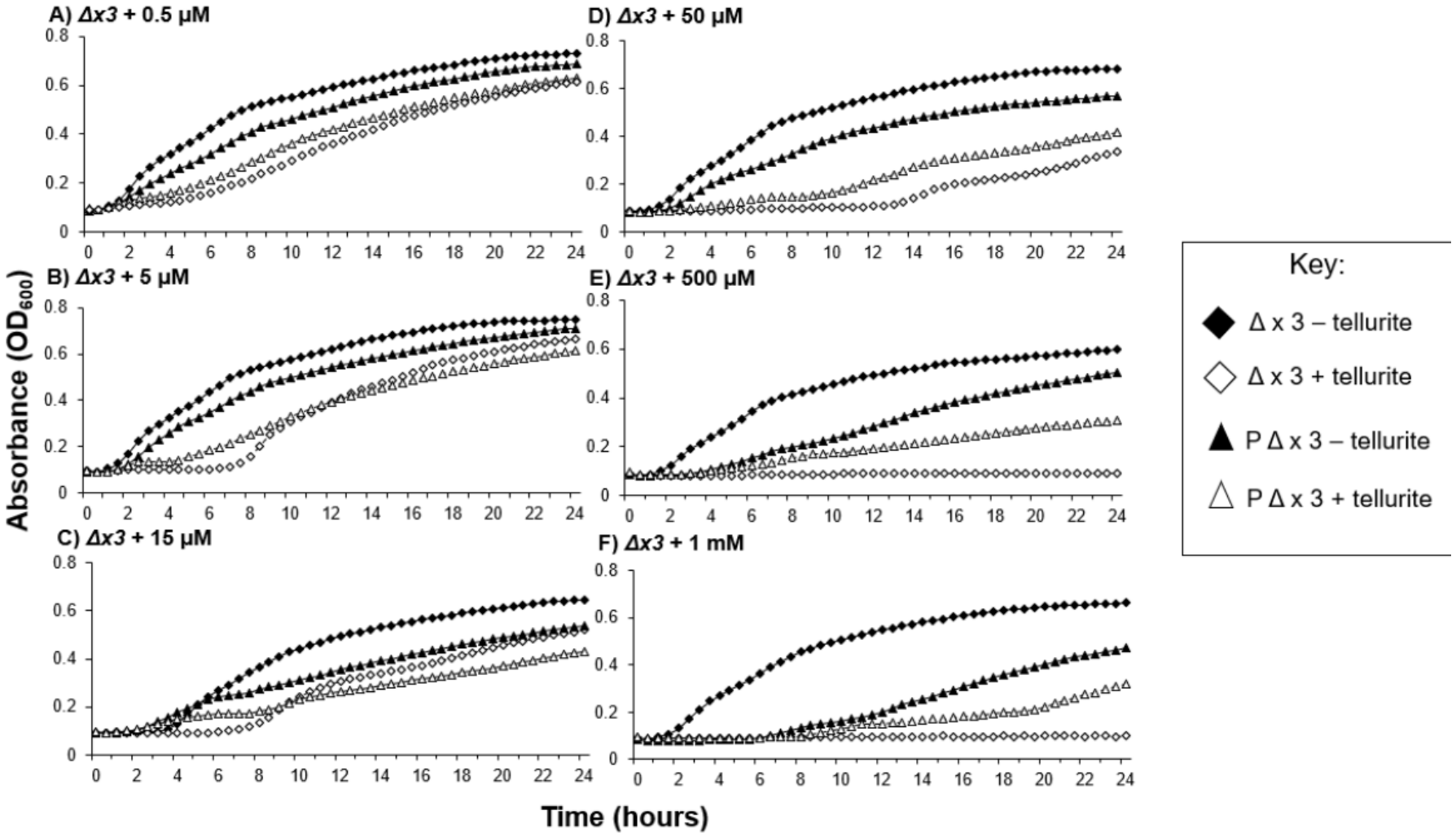


Figure 18. Generation of a tellurite resistant strain using *ΔyeaR ΔSTM1808 ΔtehB* in a directed evolution approach by passaging over 30 days in various concentrations of tellurite.
A) 0.5 µM, B) 5 µM, C) 15 µM, D) 50 µM, E) 500, F) 1 mM. Growth curves were conducted after completing passaging in select concentration (over 3-4 days) and were collected in 1 mL LB 24-well plates and optical density at 600 nm measured over 30-minute intervals over 24 hours in a SpectraMax iD5 plate reader. The Δ*x3* (diamonds) was grown alongside the passaged Δ*x3* (triangles) as a control

After generating the tellurite resistant (TR) strains their growth was compared aerobically to the original non-passaged freezer stocks. These experiments were done to prove that the passaged strains did in fact develop tellurite resistance whilst showing that the original strains did not have resistance to tellurite.

Data here confirms that tellurite resistance is present in the strain generated using directed evolution compared to the non-passaged WT. Despite increasing concentrations of tellurite, growth in the resistant strain is similar to growth without tellurite. Whereas the non-passaged strain has much lower levels of growth even in concentrations as low at 1 μM K_2TeO_3.

Growth of the non-passaged triple mutant in the presence of tellurite is virtually non-existent. However, low levels of resistance have been gained through the directed evolution method. This confirms the findings discovered so far and also highlights the growth defect observed in the passaged *ΔyeaR ΔSTM1808 ΔtehB* strain without tellurite.

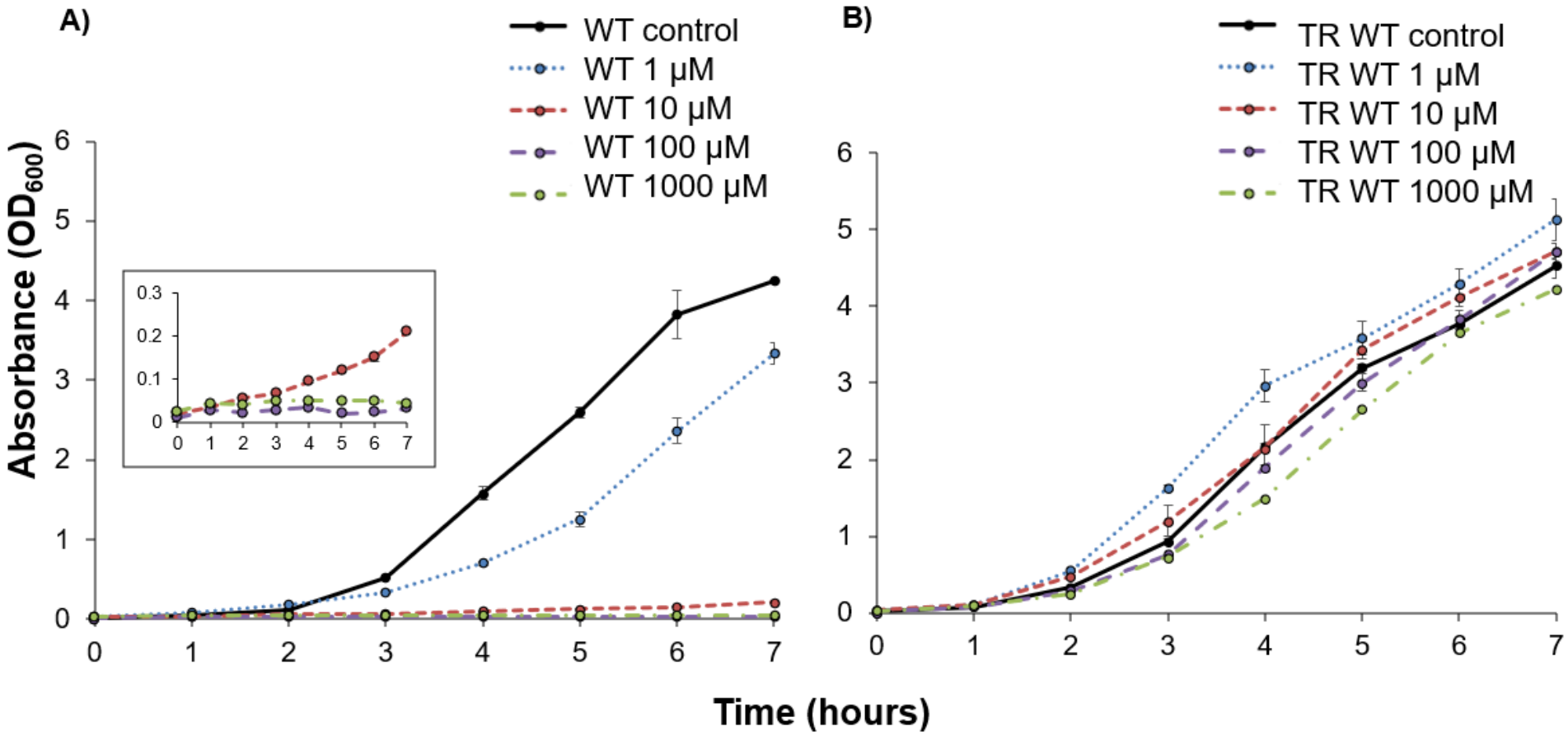


Figure 19. Passaged WT with tellurite substantially increased the tellurite resistance profile.
A) growth of WT SL1344 with 1 µM, 10 µM, 100 µM, and 1000 µM potassium tellurite and without. B) growth of passaged tellurite resistant (TR) WT SL1344 with 1 µM, 10 µM, 100 µM, and 1000 µM potassium tellurite and without. Experiments were conducted in 50 mL LB in 250 mL conical flasks at 250 rpm 37 °C. Hourly optical density readings were taken using a spectrophotometer at 600 nm over a period of 7 hours. Data is comprised of three technical repeats and standard error of the mean is plotted.

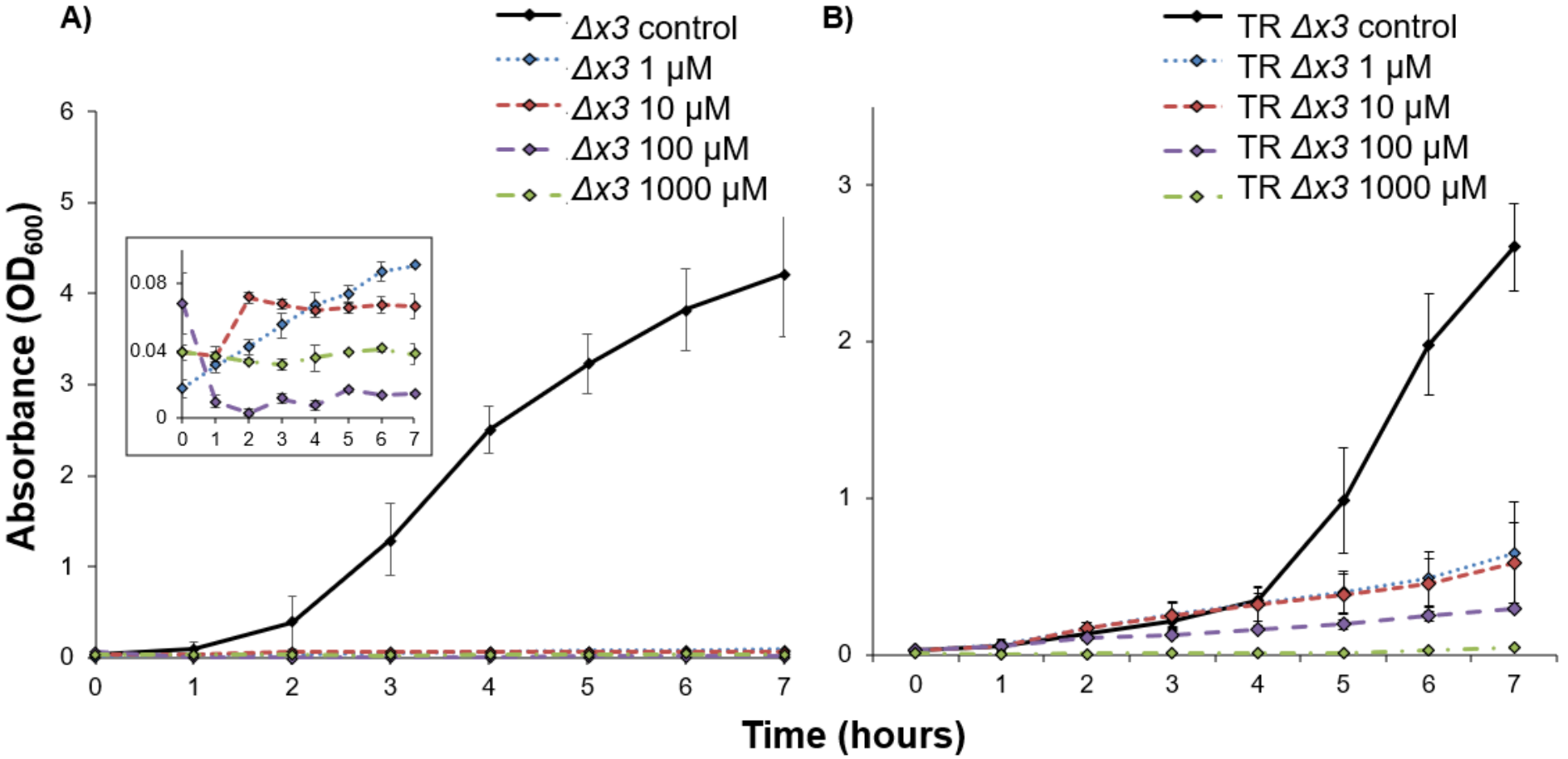


Figure 20. Passaged triple mutant has a low level of tellurite resistance at a cost of fitness.
A) growth of *ΔyeaR ΔSTM1808 ΔtehB* with 1 μM, 10 μM, 100 μM, and 1000 μM potassium tellurite and without. B) growth of passaged tellurite resistant (TR) *ΔyeaR ΔSTM1808 ΔtehB* with 1 μM, 10 μM, 100 μM, and 1000 μM potassium tellurite and without. Experiments were conducted in 50 mL LB in 250 mL conical flasks at 250 rpm 37 °C. Hourly optical density readings were taken using a spectrophotometer at 600 nm over a period of 7 hours. Data is comprised of three technical repeats and standard error of the mean is plotted

4.4.2. Tellurite resistant strains experience tellurium cytotoxicity at growth with higher concentrations of tellurite

Tellurite resistance strains possessed the ability to generate large volumes of tellurium crystallites, which resulted in a black pigment. This process was investigated to see if it was bactericidal. Measuring optical density readings of samples with high tellurium presence was predictably not as accurate as measuring typical cultures due to the change in pigment.

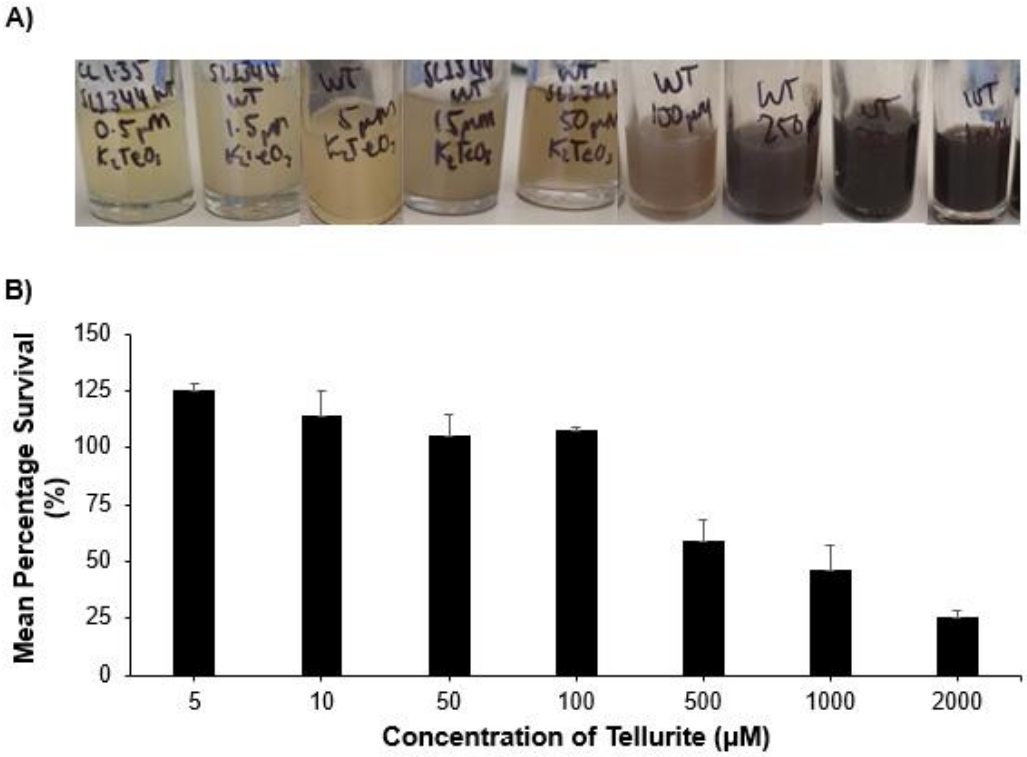


Figure 21. The generation of large quantities of tellurium crystallites in tellurite resistant WT SL1344 leads to cell lysis.
Tellurite resistant SL1344 was grown overnight in increasing concentrations of tellurite from 0.5 μM to 1 mM and photographed (A). Serial dilutions were conducted, and aliquots were spot plated on LB agar then incubated overnight to calculate Cfu/mL. Control cultures without tellurite were used to calculate the percent survival at each concentration (B). Data is composed of three biological repeats and standard error of the mean is plotted.

The pigment became darker as the concentration of tellurite in the culture increased. It is evident that the mechanism to generate tellurium is to escape tellurite mediated death; however, forming such high concentrations of this element can be lethal to the bacterium. These effects are only elicited after concentrations 500 μM, 1 mM and 2 mM as survival is much higher at the lower concentrations. These effects could only be studied in the tellurite resistant strains as the non-resistant strains would not generate enough biomass in these higher concentrations to discover this pattern.

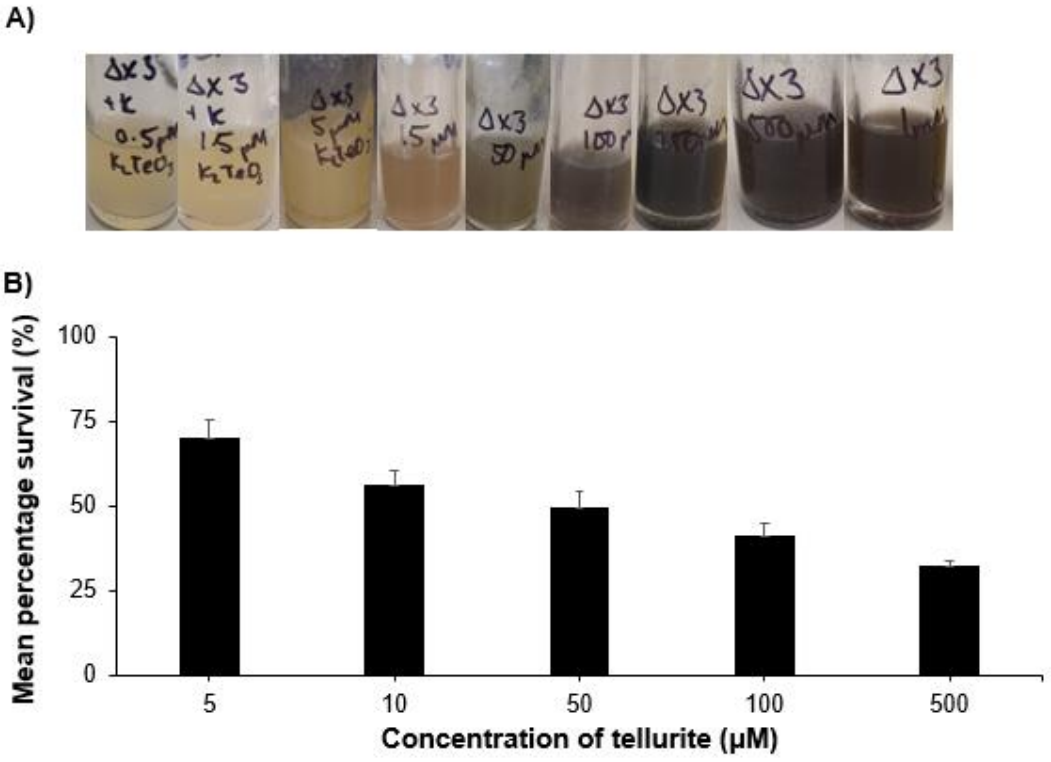


Figure 22. Tellurium crystallites are toxic to the tellurite resistant triple mutant.
Tellurite resistant *ΔyeaR ΔSTM1808 ΔtehB* was grown overnight in increasing concentrations of tellurite from 0.5 μM to 1 mM and photographed (A). Serial dilutions were conducted, and aliquots were spot plated on LB agar then incubated overnight to calculate Cfu/mL. Control cultures without tellurite were used to calculate the percent survival at each concentration (B). Data is composed of three biological repeats and standard error of the mean is plotted.

Similarly, the triple mutant with increased tellurite resistance displays the same pigmentation when exposed to higher concentrations of tellurite. The effects of tellurium are more prominent as there is lower survival in this strain than in the tellurite resistant WT. For this reason and due to time constraints, the higher concentrations were not investigated for this strain. Likewise, investigations into the non-resistant triple mutant would not be viable as it was not able to grow in these high concentrations of tellurite.

4.4.3. The NO profiles of tellurite resistant strains remain similar to non-resistant strains

Due to the interlinked nature of response systems for ROS and RNS, it was worth investigating if the tellurite resistance strains generated had altered resistance profiles for NO and H_2O_2. Gathering this information could inform how mechanisms of either ROS or RNS detoxification overlap with tellurite resistance. Genetic mutations have not been identified so the extent of change could have been extremely broad and random, only a few variables were tested below due to time constraints.

In the data gathered in NO growth curves are difficult to compare differences in growth between tellurite resistance and non-resistant strains as the growth without the NO is too variable. Calculating the growth rates allowed for relative changes in growth to be measured by fold reduction that occurs with the addition of 5 mM deta NONOate compared to controls.

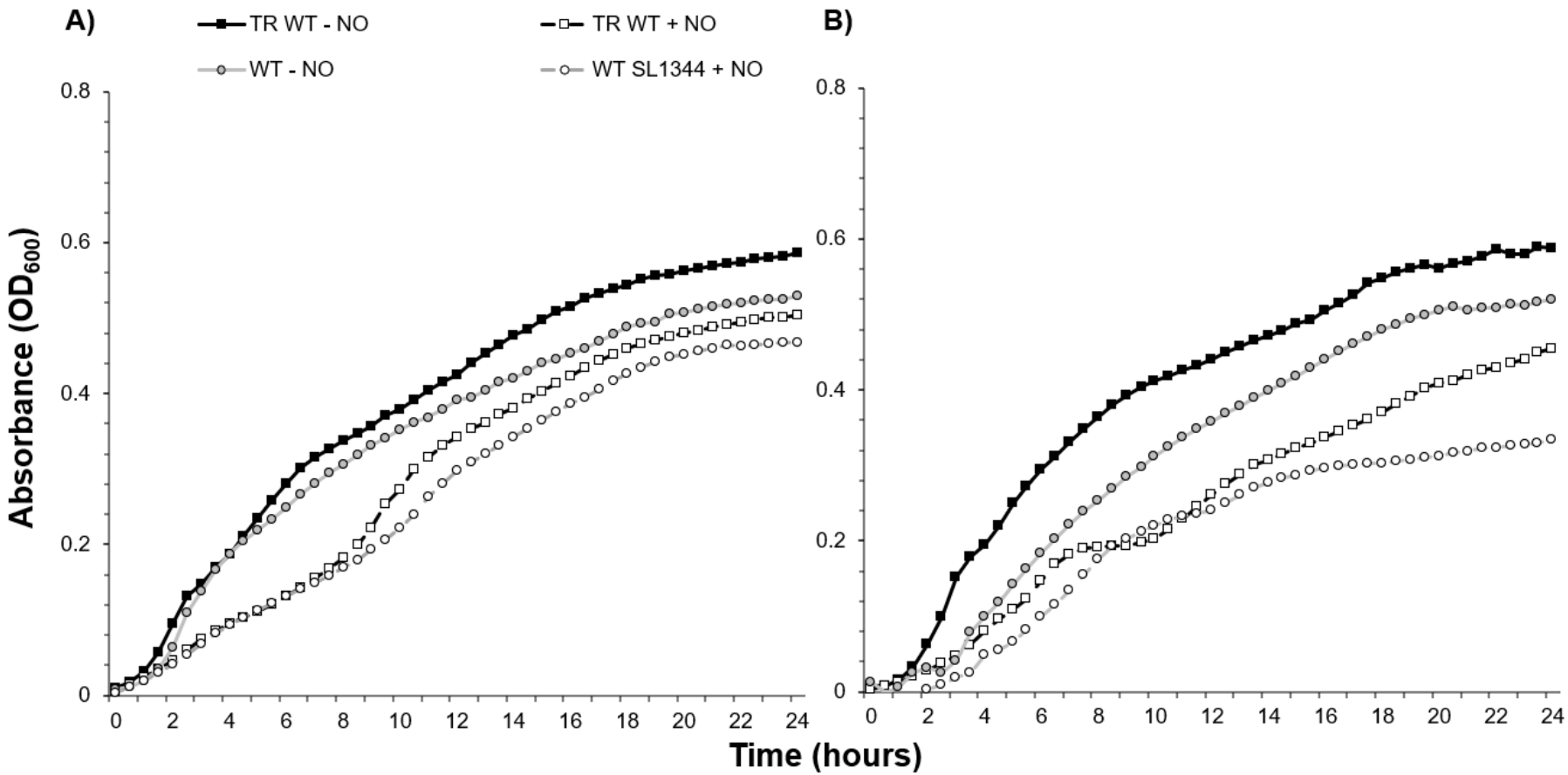


Figure 23. The addition of NO to the tellurite resistant SL1344 is more attenuating to non-resistant SL1344 anaerobically.
Optical density readings at 600 nm were plotted in 30-minute intervals over 24 hours of tellurite resistant and non-resistant WT strains with (white circles) and without exposure to 5 mM Deta NONOate (filled circles) in 1 mL LB in aerobic (A) and anaerobic (B) conditions using the SpectraMax iD5 plate reader.

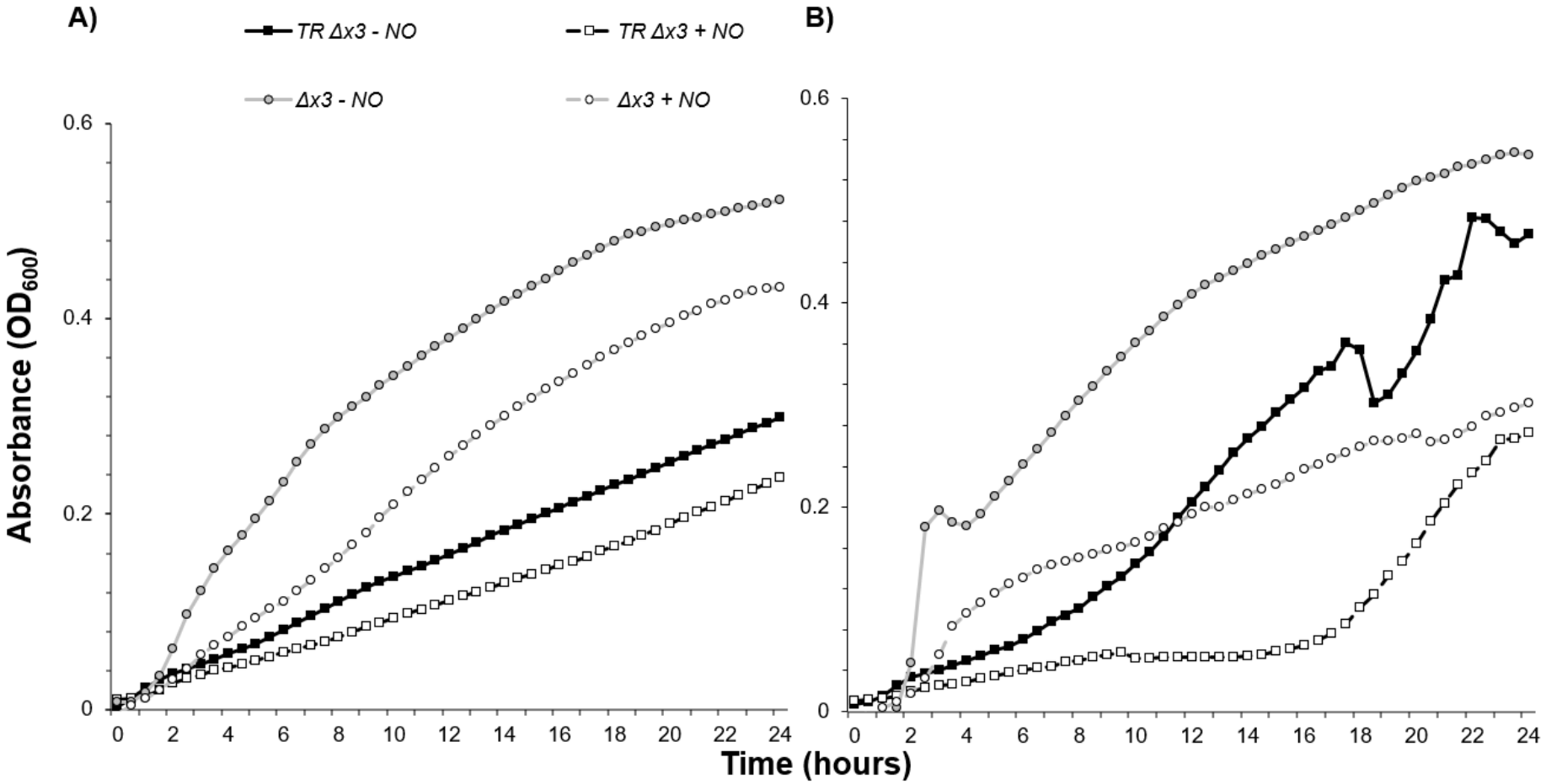


Figure 24. The tellurite resistant triple is more sensitive to NO.
Optical density readings at 600 nm were plotted in 30-minute intervals over 24 hours of tellurite resistant (squares) and non-resistant *ΔyeaR ΔSTM1808 ΔtehB* strains (circles) with (white) and without exposure to 5 mM Deta NONOate (filled) in 1 mL LB in aerobic (A) and anaerobic (B) conditions using the SpectraMax iD5 plate reader.

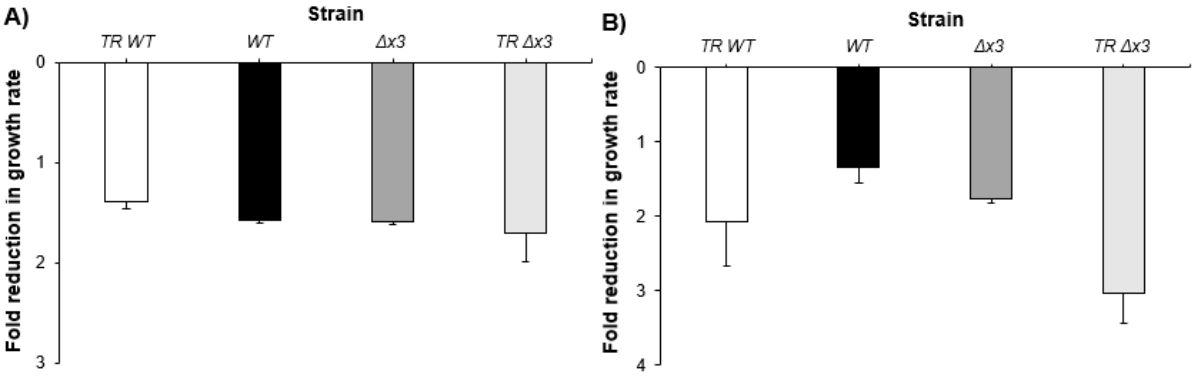


Figure 25. There are no significant differences in growth rates between tellurite resistant and non-resistant strains.
Fold change of growth rates from aerobic (A) and anaerobic (B) growth curves were calculated from natural log data seen in Figure 23 and 24 then statistically compared using ANOVAs for each growth condition, F_3=0.811, p=0.52 and F_3=3.6, p=0.06, respectively.

The growth of both strains with and without the deta NONOate are similar aerobically and the only difference observed in both conditions is an extended lag phase which constricts growth, reflected from lower OD values, during nitrosative stress. Whereas in the triple mutant with tellurite resistance there is minimal impact in growth rate during aerobic NO addition and this change is larger in the triple mutant lacking in tellurite resistance. The growth rates are dramatically reduced during NO exposure when the bacteria are no longer able to respire using oxygen. The greatest amount of change in growth was seen in the tellurite resistant strain upon exposure to the NO donor which is unable to reach exponential phase until the 17-hour mark. It must be stated that as the experiment continues, the amount of NO presence decreases as the donor compound has a relatively short half-life at 37 °C and this may allow for increase in growth at later stages.

It is interesting that the tellurite resistant strain generated from the WT SL1344 has better growth in general compared to its non-resistant counterpart and that this is completely reversed in the triple mutant. Although a pattern emerges between the two whereby growth is not as attenuated during aerobic conditions and this depreciation in growth is amplified during anaerobic conditions. These tellurite resistant strains were generated only during exposure to oxygen and this situation may have had an impact on the mutations acquired that could overlap to other metabolic processes. This is because oxidative stressors which are major causes of mutations are more prevalent in the presence of oxygen.

4.4.4. Resistance to hydrogen peroxide is altered in tellurite resistant strains

The results of NO exposure to tellurite resistant strains were diverse so these strains were tested against oxidative stress using hydrogen peroxide in several assays and typical 24-hour aerobic and anaerobic growth curves.

Typically, *Salmonella* have a natural resistance to H_2O_2 as they have designated response mechanisms. The pattern observed from a timed assay confirms that WT SL1344 possess some tolerance to the ROS, but survival is drastically lowered after 2-3 hours of exposure. However, survival in the tellurite resistant strain is much higher after 2 hours of treatment with 20 mM H_2O_2 and although a drop is seen, the level of survival is higher after 3 hours compared to the WT SL1344. This would suggest that this strain has developed a higher tolerance to hydrogen peroxide which has been acquired during passage in the presence of tellurite. Similarly, disc diffusion assays with various concentrations of H_2O_2 showed that the tellurite resistant strain had much lower areas of inhibition compared to WT signifying an enhanced resistance to the molecule.

As both of these assays were conducted aerobically, growth curves were completed both aerobically and anaerobically with and without the presence of 10 mM or 100 mM H_2O_2. Gaining this data would allow a more complete picture of the phenotype present. Remarkably, the addition of 10 mM H_2O_2 improved the rate of aerobic growth up to 12% for both WT strains. The most interesting observation is the 100 mM H_2O_2 addition which generates an extended lag phase lasting up to 5 hours for the tellurite resistant strain in aerobic conditions giving a 3% reduction in growth rate and 8 hours anaerobically giving 71% reduction in growth rate. However, no optical density readings were legible with 100 mM H_2O_2 in both conditions for the WT SL1344 strain which had tellurite sensitivity. Although growth was capped more anaerobically at 100 mM, growth with 10 mM surpassed the growth rate without H_2O_2 by 30% for the tellurite resistant strain. This may be because increased resistance being mediated by a catalyse that favourably operates aerobically. In comparison, the non-modified WT strain had a 16% increase in growth rate anaerobically during 10 mM H_2O_2.

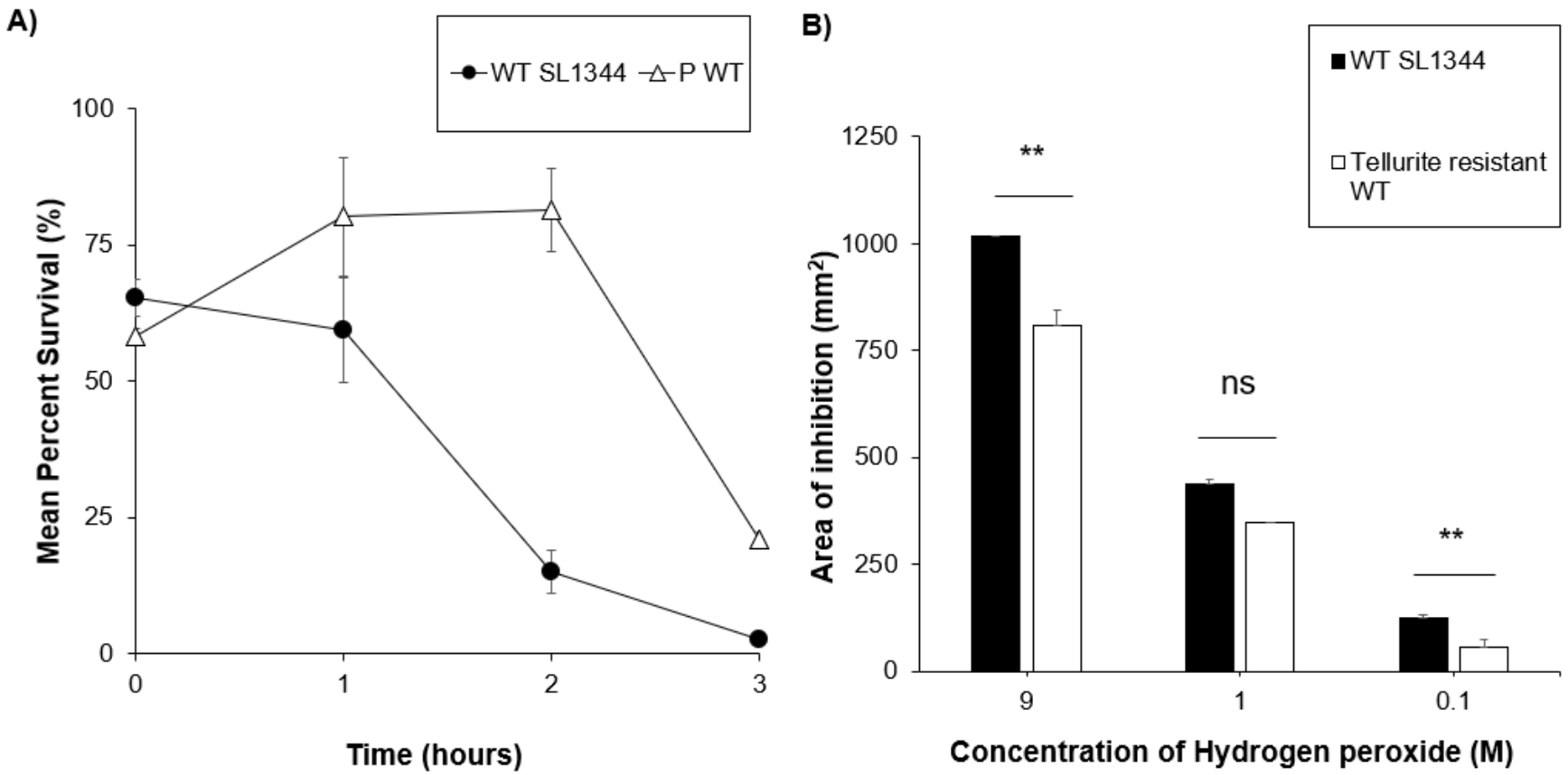


Figure 26. Tellurite resistance in the WT background invertedly causes increased hydrogen peroxide resistance.
Survival of WT and passaged tellurite resistant (P WT) strains during 3 hours of 20 mM hydrogen peroxide treatment (A). Data is composed of two technical repeats with three biological repeats and standard error is plotted. B) area of inhibition from hydrogen peroxide disc diffusion assays. Data is composed of three technical repeats with standard error indicated in bars. ** p<0.01, ns = non-significant.

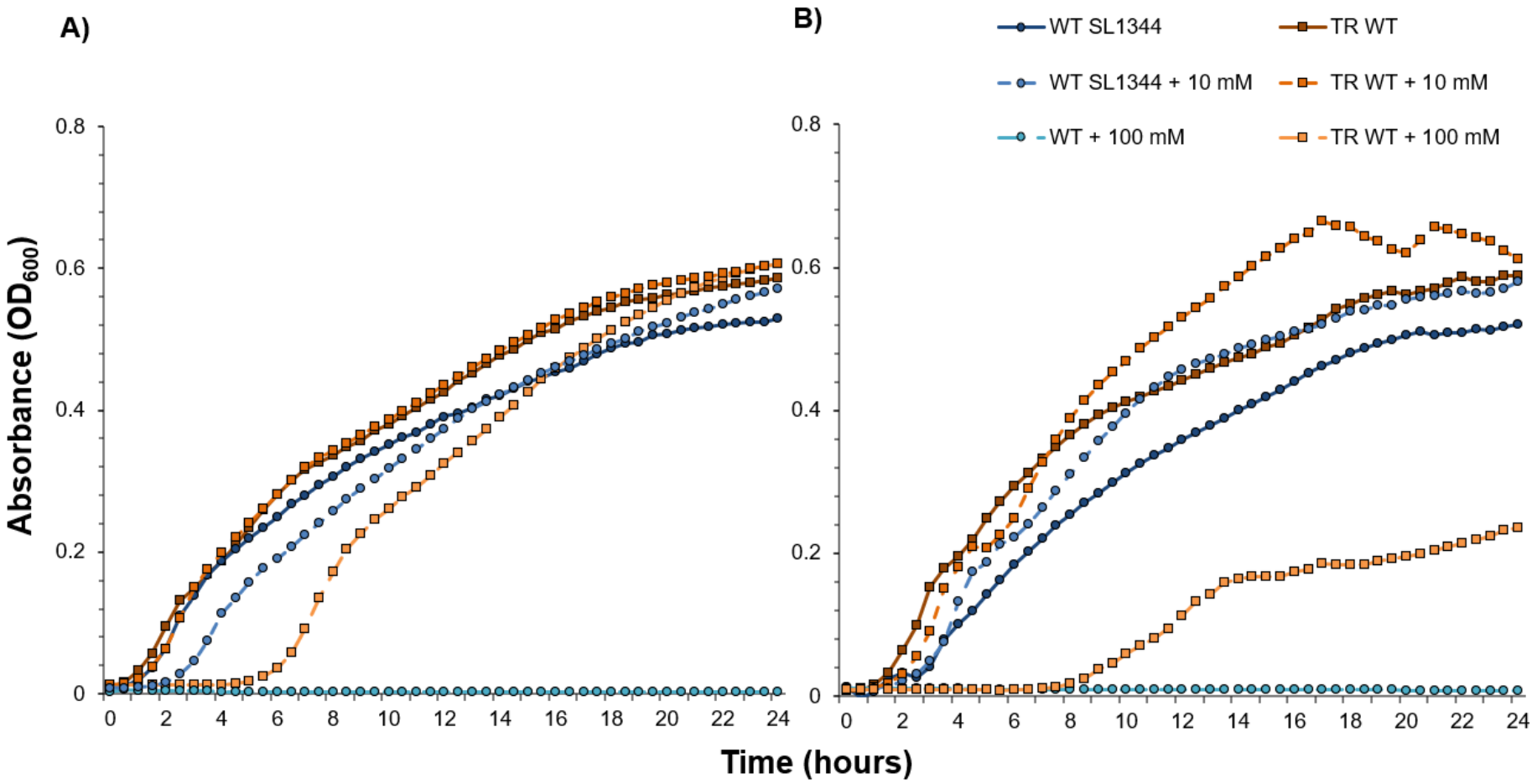


Figure 27. Resistance to 100 mM H_2O_2 is more prominent in aerobic conditions of the WT tellurite resistant strain.
Growth curves with tellurite resistant (TR) WT strain (squares) and non-resistant WT SL1344 (circles) during exposure to 0 mM, 10 mM and 100 mM concentrations of hydrogen peroxide in 1 mL LB. This was conducted in aerobic (A) and anaerobic (B) conditions using the SpectraMax iD5 plate reader which measured OD readings at 600 nm in 30-minute intervals over 24 hours.

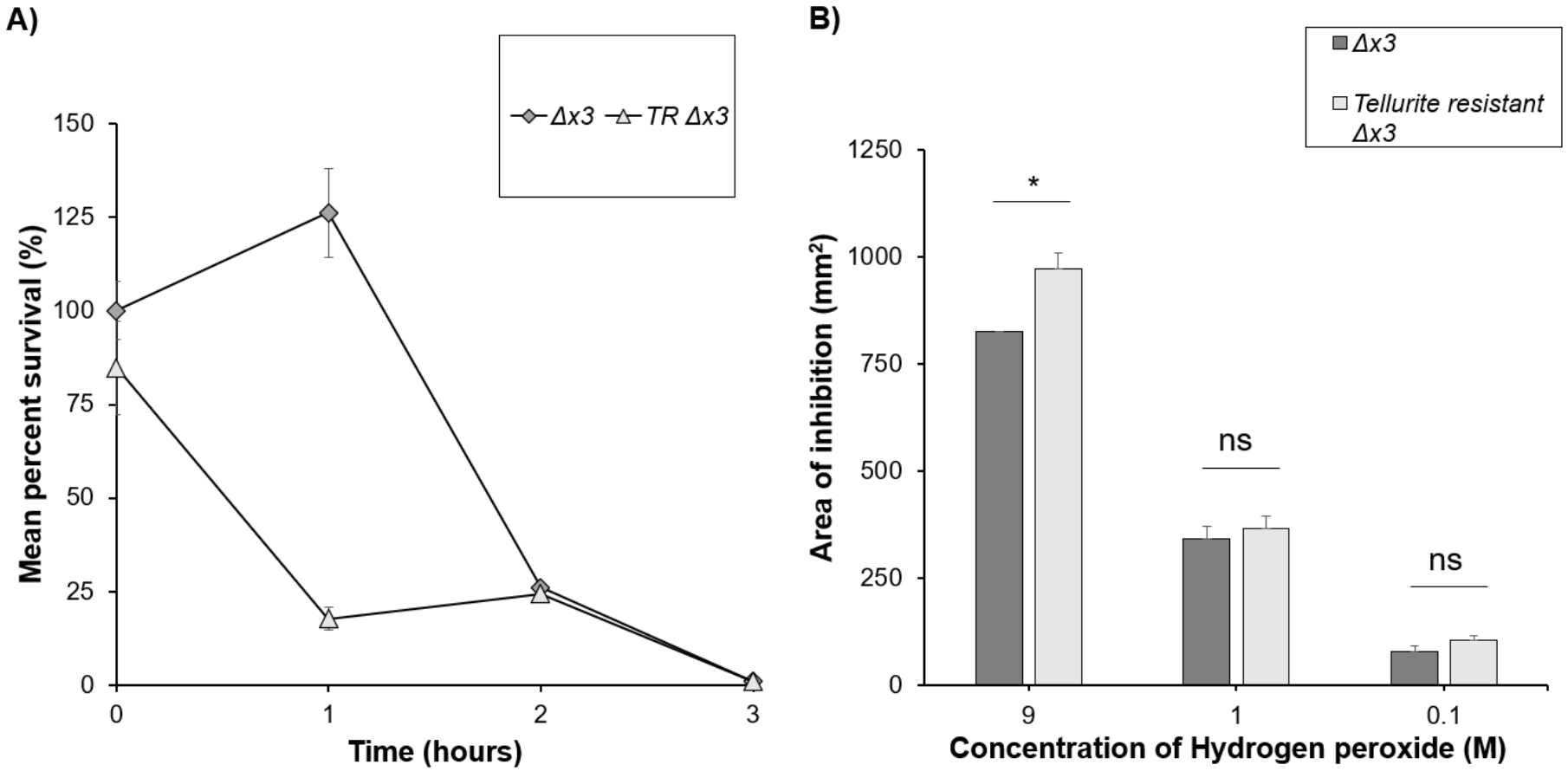


Figure 28. Higher susceptibility to H_2O_2 is seen in the tellurite resistant triple mutant strain.
A) Survival of *ΔyeaR ΔSTM1808 ΔtehB* and passaged tellurite resistant strains during 3 hours of 20 mM hydrogen peroxide treatment. Data is composed of one technical repeats with three biological repeats and standard error is plotted. B) area of inhibition from hydrogen peroxide disc diffusion assays. Data is composed of three technical repeats with standard error indicated in bars. * $p < 0.05$, ns = non-significant.

The *ΔyeaR ΔSTM1808 ΔtehB* tellurite resistant strain was investigated to assess if that also gained higher resistance to hydrogen peroxide. Although genetic changes for this strain had not been investigated so any mutations acquired that gave tellurite resistance may be different to those seen in the tellurite resistant WT strain. Controversially, the *Δx3* tellurite resistant strain experienced a lower survival rate after 1 hour of exposure to 20 mM H_2O_2 in comparison to the non-resistant strain. The differences reduced at 2 and 3 hours giving relatively similar survival rates. This data suggests that this tellurite resistant *Δx3* strain developed sensitivity to H_2O_2 which is the opposite effect to the WT tellurite resistant strain, supporting the fact the tellurite resistance eliciting mutations differ between the strains.

Growth curves were conducted to assess the addition of 10 mM and 100 mM H_2O_2 on the triple mutants to see if this supported findings of H_2O_2 sensitivity in the tellurite resistant strain. This data allowed the differences in tolerance for ROS to be compared in aerobic and anaerobic conditions. Aerobically, the addition of 10 mM H_2O_2 results in 38% loss in growth rate in the *Δx3* mutant but this is only 33% reduced in the tellurite resistant *Δx3*. By increasing the concentration to 100 mM, both strains have no readable OD_{600} readings resulting in growth rates plummeting 100% regardless of oxygen presence. Interestingly, during anaerobic conditions, the growth rate of the *Δx3* mutant increases by 20%. However, this is reduced by 35% in the tellurite resistant *Δx3* strain. The lack of growth in the tellurite resistance strain is noted again here during aerobic and anaerobic conditions as is likely due to deleterious mutations obtained during the directed evolution process.

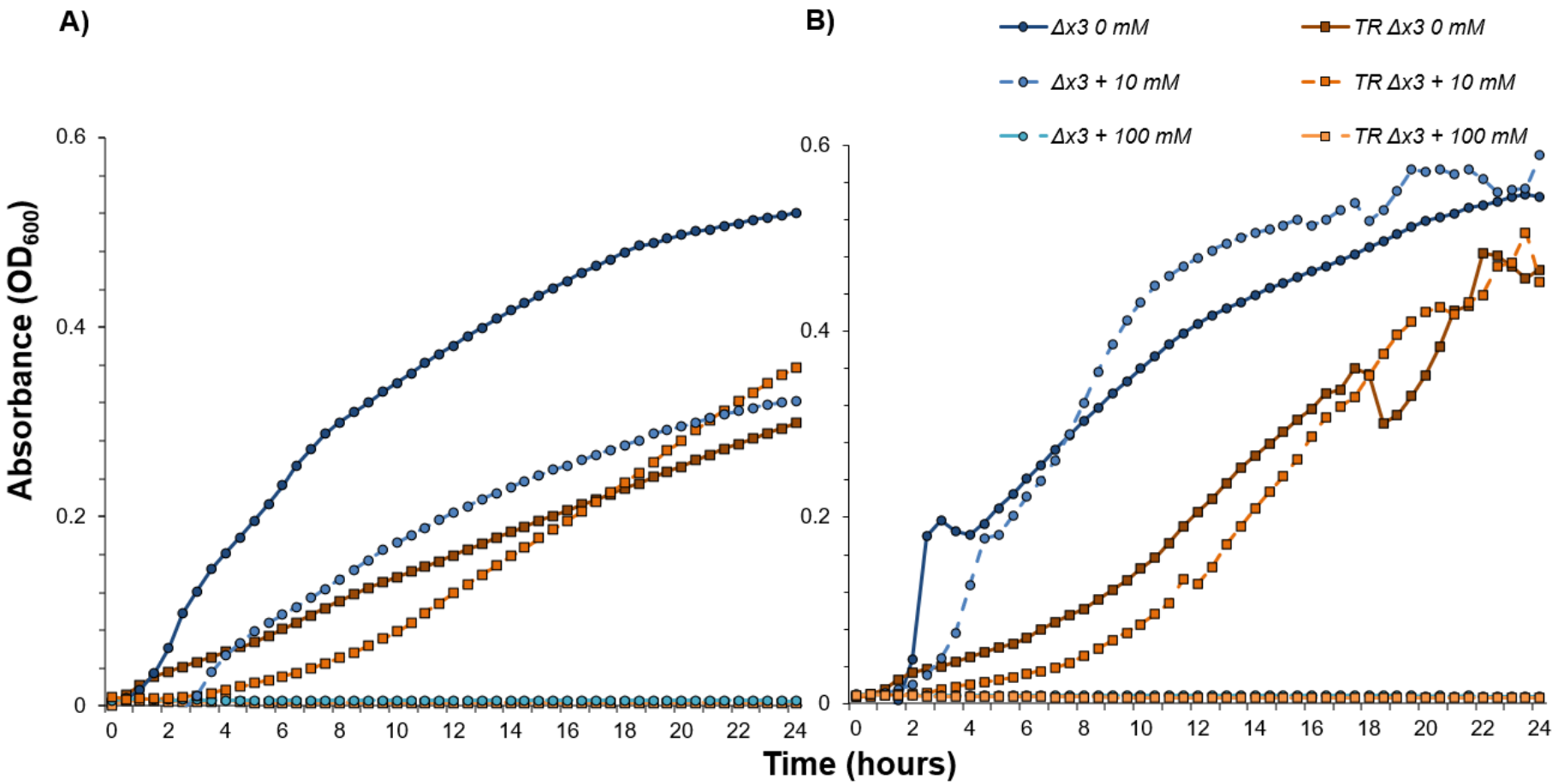


Figure 29. Resistance to H_2O_2 was not gained in the triple tellurite resistant strain.
Growth curves with passaged triple knockout tellurite resistant strain and non-passaged triple knockout strain exposure to 0 mM, 10 mM and 100 mM concentrations of hydrogen peroxide in 1 mL LB. This was conducted in aerobic (A) and anaerobic (B) conditions using the SpectraMax iD5 plate reader which measured OD readings at 600 nm in 30-minute intervals over 24 hours.

4.4.5. Trading tellurite resistance for antibiotic sensitivity

The commonality of heavy-metal resistance being prominent with antimicrobial resistance justified further investigation of other phenotypes in the tellurite resistant strains developed. Although tellurite resistance was developed through the directed evolution of SL1344 so additional mutations could be a direct result of passaging cells and random acquired single nucleotide polymorphisms which in turn could be gain or loss of function in some genes. Consequently, other phenotypes would be developed including antimicrobial resistance or even sensitivity.

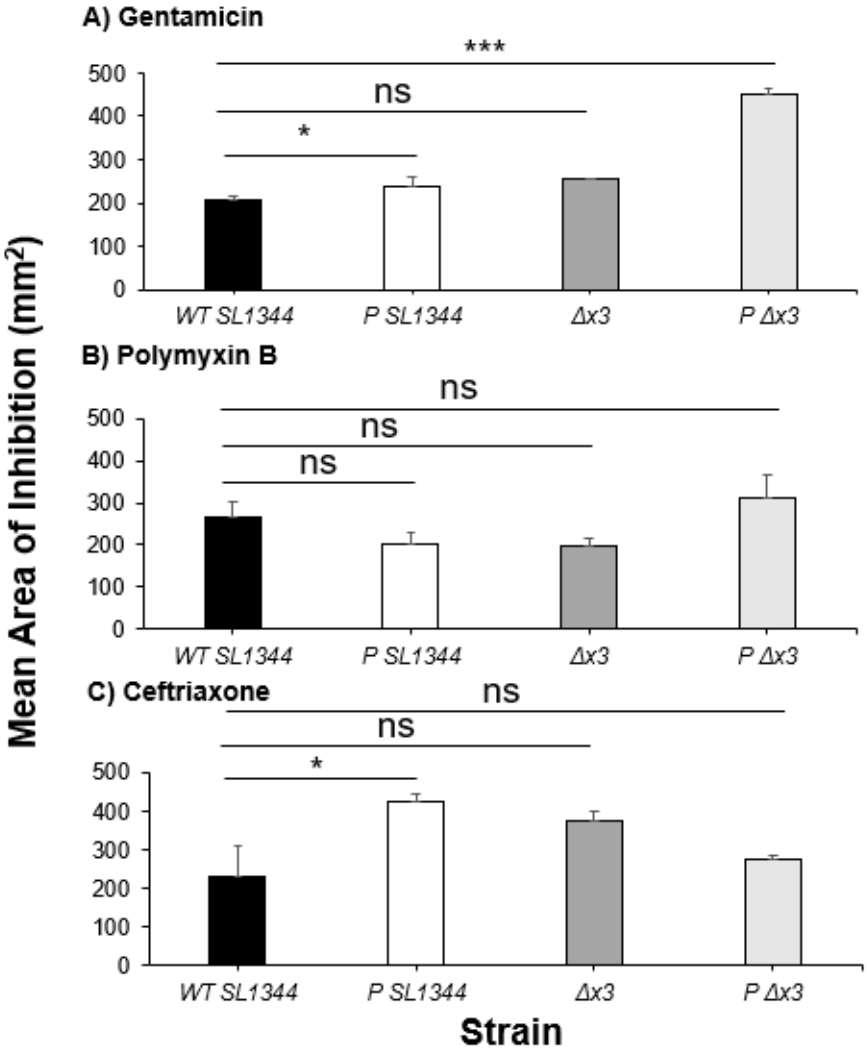


Figure 30. Increased sensitivity to gentamicin and ceftriaxone occurs in the tellurite resistant strains.
Diameter of clear zones was measured to calculate area of inhibition for 50 mg/mL Gentamicin (A),10 mg/mL Polymyxin B (B) and 1 mg/mL Ceftriaxone (C). Data displayed consists of three technical repeats and standard error is plotted. Statistical analysis using ANOVA and Tukey's multiple comparisons test. *** indicates $p < 0.001$ and * $p < 0.05$, ns = non-significant.

The level of inhibition in WT was used to compare the differences seen in the tellurite resistance strains and the *Δx3* mutant. The WT tellurite resistant strain had a significantly larger zone of inhibition than WT which would mean an increased sensitivity to Gentamicin and Ceftriaxone. These broad-spectrum antibiotics have different targets within bacteria but they both bind to proteins causing subsequent inactivity. Additionally, Gentamicin and Polymyxin B sensitivity is evident in the *Δx3* tellurite resistant strain despite no significant differences observed against WT. Although differences to inhibition zones were not significant for Polymyxin B there was more growth on the WT tellurite resistant strain showing this could be more resistant to this antibiotic, but this requires more investigation. The *Δx3* strain had no significant differences compared to WT in inhibition zones for each antibiotic and they are relatively similar for Gentamicin and Polymyxin B, but much larger with Ceftriaxone. It would be worth investigating further as currently the standard error is high which doesn't allow differences to be highlighted.

4.4.6. Survival within macrophages is no different in the WT tellurite resistant strain

Conducting gentamicin protection assays in RAW 264.7 macrophages tested the hypothesis that altered tellurite resistance can impact survival within macrophages due to the additional changes the mutations have caused to give certain ROS and RNS phenotypes. The change in dealing with NO differed between WT and its tellurite resistant strain aerobically where the tellurite resistant strain grew better, this was reversed anaerobically and for H_2O_2 a similar pattern was seen. As surviving in macrophages require all of these features to be enhanced especially in a microoxic environment, it could be expected that little differences or lower survival would be seen in the tellurite resistant strain.

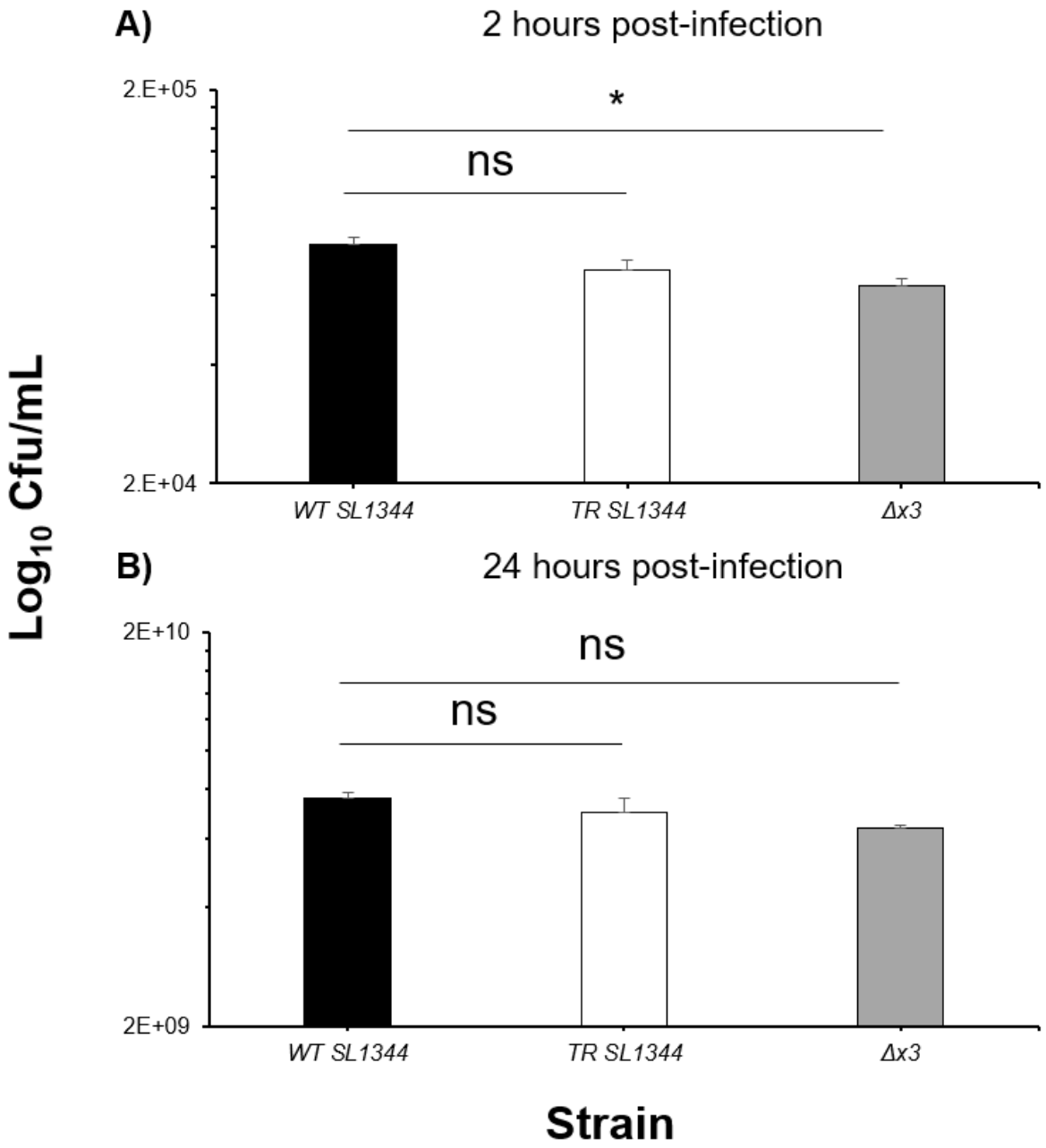


Figure 31. No differences are seen in bacteria survival in macrophages between the tellurite resistant and parent WT strain.
Untreated RAW 264.7 macrophages were infected with WT SL1344, tellurite resistant WT and *ΔyeaR ΔSTM1808 ΔtehB* in a multiplicity of infection of 10:1 and incubated for 2 hours (A) and 24 hours (B). Macrophages were lysed with 1% Triton X-100 and the cell lysate was serially diluted to allow Cfu/mL calculations and the $\log_{10}$ was plotted for each strain. The data was composed of three biological repeats with SEM plotted and normalised to a dose of 10^6 *Salmonella*. Statistically compared using ANOVA at 2 hours (F_2= 6.99, p= 0.02) and 24 hours (F_2= 2.6, p= 0.15) with Tukey's multiple comparisons test. Asterisk (*) signifies p<0.05 and ns = non-significant.

After 2 hours of infecting the murine macrophages, differences were observed in bacterial cells recovered denoted as the Cfu/mL. Significantly lower amounts of *Δx3* mutants were recovered (p=0.02) meaning that this strain was less capable of infecting the macrophages. The tellurite resistant WT strain did not significantly differ from WT (p= 0.13) despite a reduced Cfu/mL recorded. At 24 hours post infection, similar Cfu/mL values were obtained from all strains meaning there was no significant attenuation in macrophage survival in the tellurite resistant strain and the *Δx3* compared to WT (p= 0.54 and p= 0.13). Notably, the macrophages used were not pre-exposed to stimulating cytokines which may have altered the survival seen for these strains.

4.5. Discussion

The toxicity of tellurite which has been explored previously in Chapter 3 highlights the need for detoxification strategies in bacteria that may encounter the metalloid. Despite *Salmonella* not likely being exposed to tellurite, they contain low levels of resistance to the molecule. Furthermore, resistance can be extrapolated to much higher concentrations for both WT and to low levels in *ΔyeaR ΔSTM1808 ΔtehB*. The gain of tellurite resistance did not impact the fitness of the WT strain but the same cannot be said for tellurite resistance in *ΔyeaR ΔSTM1808 ΔtehB*. This is likely due to accumulation of such deleterious mutations that may have impacted other important metabolic functions (Heilbron et al., 2014) or reallocation of energy to maintain metabolic changes leading to reduced growth (Anand et al., 2019). It is also worth mentioning that it seemingly displayed the small colony variant phenotype (not shown) which can occur during evolution studies, especially to favour biofilm formation in liquid cultures. Moreover, the tellurite resistant triple strain displayed less resistance than previously recorded when isolated into single colonies. Bacterial biofilms are complex structures of non-motile bacteria which usually attach to surfaces in a protective extracellular matrix composed of extracellular polymeric substances. A unique trait of biofilms compared to planktonic bacteria, is that they are consistently more resistant to various stressors such as antibiotics and heavy metals (Hall and Mah, 2017). This may explain what occurred in the plate reader when the non-passaged strains superficially displayed resistance.

The resistance method has not been genetically investigated here; however, phenotypically the production of tellurium crystallites were visible. Interestingly, the black pigment is not the true natural colour of elemental tellurium, it is typically a silvery white colour (Doerner, 1922). This contradicts when the element is microbially generated. This issue has not truly been addressed in the literature, but many studies highlight the production of tellurium being a dark black pigment and the closely related, selenium being red (Zannoni et al., 2007). When tellurium is within bacterial cells it can induce changes to the cell membrane (Kim et al., 2012),

alter glutathione metabolism, substitute other metals in proteins and even cause oxidative stress (Valdivia-González et al., 2012). Other authors have previously discussed the idea that reduction of tellurite consequently leads to cell death due to cost of thiols such as cellular glutathione which put metabolic processes under arrest (Taylor, 1999). The present work thereby supports the assumption that tellurium is in fact toxic and proposes the production of tellurium is a suicide function of bacteria rather than a response that has developed to rescue the bacteria. In addition to the toxicity elicited by tellurium, the element can also be re-oxidised in the presence of oxygen which can cause further issues. Therefore, potentially higher concentrations of tellurium would be found in resistant cells grown in anaerobic conditions. This has not been investigated in this study so could be conducted in further studies. Previous investigations in tellurite detoxification suggest that deposition of tellurium crystallites is exclusively intracellular. Furthermore, transmission electron microscope studies support that the metalloid is found in close proximity to cell membranes (Zannoni et al., 2007). This has brought about the argument of whether tellurium can be transported as it has been found in surrounding media, but this could simply be due to cell lysis. Data collected here supports that high concentrations of tellurium can result in cell lysis, and it is unlikely that *Salmonella* possesses any tellurium transport proteins.

In some aerobic photosynthetic bacteria, tellurite resistance does not depend on tellurium accumulation (Maltman and Yurkov, 2019). The chalcogenides can be detoxified using a glutathione-based reaction as described by Turner et al. (2001). Alternatively, it could rely on catalytic proteins which are located in the periplasm or cytoplasm, for example cytoplasmic oxidoreductase (Avazéri et al., 1997). Another method of detoxification described by Zawadzka et al. (2006) details a siderophore present in *Pseudomonas stutzeri* which is able to precipitate and thereby reduce selenium and tellurium oxyanions. The final method is the direct or indirect reduction using electrons generated through the respiratory chain (Trutko et al., 2000). Resistance could even be mediated by preventing transport of tellurite into cells which occurs in *E. coli* with mutated phosphate and acetate transporters (Elías et al., 2015). Adaptive resistance can often lead to reverting, due to its transient nature upon removal of the inducing factor which could be another explaining factor in the varied resistance in the tellurite resistant triple mutant. However the accumulation of mutations over long periods can lead to defined changes in genetic material which are considered more stable and can be passed on to subsequent progeny (Vávrová et al., 2021). This is a probable explanation to the resistance generated in the tellurite resistant WT strain.

As this project is comparing tellurite resistance with NO detoxification, the next logical step was to test the differences in NO profiles for the tellurite resistant strains. The data collected here in NO growth curves were not comparable as the growth between the strains were too

variable without the NO donor. This can occur in the plate reader which gives natural variation, but it can also be a consequence of mutations impacting the strains ability to grow in certain environments. Therefore, fold change upon NO donor addition was calculated using growth rates with and without the presence of NO. The analysis yielded no significant differences in growth rates upon exposure to NO aerobically. This was also the case anaerobically, but growth of both tellurite resistant strains suffered upon addition of the deta NONOate more so than non-resistant parent strains. This could be due to mutations present in anaerobic NO detoxification systems such as NrfA, NorVW or Hmp which has allowed these proteins to combat tellurite stress instead of RNS. Alternatively, the tellurite resistant strains could contain mutations in transcriptional regulators of these NO detoxification genes. It is unlikely though that mutations would form in places that would be detrimental to survival in other aspects. Furthermore, anti-mutation systems in bacteria are extremely effective at minimising spontaneous mutations. However, these strains were only exposed to one stressor at high concentrations (tellurite) which induces ROS enabling them to be more evolved for dealing with tellurite stress instead of nitrosative stress by overwhelming DNA repair pathways. The environment which the facultative anaerobe is grown in is important during directed evolution. This was proven by Shewaramani et al. (2017) and colleagues when they discovered *E. coli* had higher levels of diverse spontaneous mutations when grown anaerobically.

Work by Toptchieva et al. (2003) found that the *ter* operon for tellurite resistance in *Proteus mirabilis* had low levels of expression during oxidative stress by H_2O_2 in addition to tellurite. The targeted mutation of serine to tellurocysteine in glutathione actually adapted the enzyme to catalyse hydroperoxides (Liu et al., 2009). Other studies have linked ROS to tellurite as this toxic metalloid elicits an oxidative stress which is why resistance to H_2O_2 was assessed in the tellurite resistant strains (Turner et al., 1999, Turner et al., 2001). ROS commonly target DNA, nucleotides, amino acids, lipids and vitamins which can contribute to mutation formation or impact metabolic functioning (Rhen, 2019). Due to the intrinsic link between tellurite and ROS, it was hypothesised that a tellurite resistance phenotype would pair suitably with increased resistance to H_2O_2 either by increased thiol production or overexpression of detoxification enzymes. This was indeed found in the WT evolved tellurite resistant strain which was more prominent during aerobic than anaerobic growth conditions which could point to changes in superoxide dismutases. However, the same could not be said for the triple tellurite resistant strain which appeared more sensitive to H_2O_2 than its non-resistant parent strain which was consistent with or without the presence of oxygen.

Salmonella have several catalyses (KatE, KatG, KatN) and peroxidases (AhpC, Tpx, TsaA) which degrade H_2O_2 into molecular water and oxygen (Rhen, 2019). These are controlled by SoxRS and OxyR transcriptional regulators which also play roles in other envelope stress

responses. Reasons as to why only one tellurite resistant strain has higher H_2O_2 tolerance could be due to the different mutations acquired during directed evolution. Mutations could be present in the transcriptional regulators which consequently overexpress ROS genes which can lead to resistance. This is seen in Anand et al. (2019) study of the transcriptional activator OxyR in *E. coli*. They found mutations present in a conserved region to gain oxidative resistance was common and found a trade-off for growth rate to gain this tolerance. Earlier studies also confirm that mutations can be found in OxyR which lead to peroxidase resistance (Mongkolsuk et al., 2000). Similarly, mutating SoxRS does not give as strong of a hydrogen peroxide phenotype and has been recorded to be more important in superoxide stress survival (Daugherty et al., 2012). Therefore, investigating other ROS may be useful to assess other potential modifications.

The development of antibiotic resistance by acquiring resistance genes is not always down to selective pressure to gain resistance, but a product of gaining resistance against other selectable markers (Granato et al., 2019). This would have been the case if antibiotic resistance would have been detected here, but in fact there is sensitivity to gentamicin and ceftriaxone in tellurite resistant strains and inconclusive for polymyxin B. Antibiotic resistance to clinically relevant drugs is a common phenotype to acquire through genetic mutations as it is favourable to pathogen survival (Alcaine et al., 2007). Moreover, antimicrobial sensitivity is less common but can still occur in laboratory grown strains (Liu et al., 2010). Cross resistance arises when spontaneous mutations or acquired resistance genes that occur for the treatment drug have a positive or negative impact on another drug (Baym et al., 2016). Therefore, collateral sensitivity can occur during evolution with a treatment and result in resistance to one but sensitivity to another treatment. In this study for example, this could mean increased resistance to tellurite and sensitivity to gentamicin. Potentially the same explanation could apply to NO and H_2O_2 phenotypes. Another explanation could be that level of resistance to certain compounds reside in dormant cells like persisters. Persisters are multidrug tolerant cells composed of unstable, non-growing bacteria that are a result of phenotypic switching. Typically, formation of these cells are related to acid exposure and carbon starvation. Moreover, despite tolerance being high due to survival in high stress situations, the progeny of persisters usually contain antibiotic sensitivity (Helaine et al., 2014).

In order for macrophages to produce damaging RNS using iNOS, they require stimulation from either microbial products or cytokines such as IFN-γ, TNF-α and IL-1 (Lee et al., 2000). All of which are important for *in vivo* macrophage migration to the site of infection, subsequent activation and RNS/ROS-mediated killing (Aktan, 2004). The *Salmonella* containing vacuole within the macrophage that houses the bacteria once invaded is not free from threats. Although presence of Sif proteins surrounding the vesicle prevent fusion of lysosomes that

are highly acidic and microoxic among other stressors (Uchiya et al., 1999, Brumell et al., 2001). The murine macrophages used in this study were not activated using any of these cytokines which would impact the survival rates seen here as fully lethal concentrations of RNS and ROS would not have been obtained. Thus, lower concentrations of these reactive radicals were produced in the macrophage which would only have a bacteriostatic effect which could explain the similar levels of survival. When *Salmonella* are internalised in macrophages they can form different cell types. Within the subpopulations are persister cells, metabolically active or metabolically inactive cells that may have the ability to resume replication post exocytosis. This enables the pathogen to effectively combat the next stage of infection (Helaine et al., 2014).

Further work could be continued to characterise the genetic mutations caused by the directed evolution in both tellurite resistant strains. Whole genome sequencing (WGS) is being conducted to compare strains developed to the parental reference genome. Bioinformatic analysis could allow the identification of SNPs, frameshift mutations, altered non-coding regions such as promoters or synonymous mutations as it would help to inform the phenotypes seen here. Mutations can accumulate naturally over time through genetic drift so the mutations may not all contribute to the tellurite resistance, or any other phenotype seen here, but improve the fitness or survival of the microbe. A way to overcome this would be repeating the study and isolating the cultures after each passage and having this sent for WGS. This would pinpoint the SNPs occurring and enable the assessment of how likely each mutation would contribute to causing a deleterious effect or perhaps a gain of function like resistance. Another unknown is whether passaging strains exposed to tellurite is the reason for resistance developing. It would be worth evaluating whether tellurite resistance could be developed in a strain that is passaged for the same period of time but without exposure. Furthermore, the selection pressures of anaerobically grown strains with tellurite should be investigated further. More empirical data could be obtained about the NO, H_2O_2 and antibiotic phenotypes to support or disprove these findings.

Chapter 5: Investigating the role of YgbA in tellurite resistance, NO detoxification and macrophage survival

5.1. introduction

As previously described, NO is a free radical that can be generated by iNOS in macrophages due to microbial products and cytokine activation (Chandrasekar et al., 2013). During *Salmonella* infection, macrophages receive stimulation through detecting LPS or via pro-inflammatory cytokines such as IFN-γ, TNF-α and IL-1 and 6 (Aktan, 2004, Yadav et al., 2018). The stimulation that macrophages receive can impact the amount of ROS and RNS produced, which can impact the effectiveness of macrophage mediated killing. The molecule can also form naturally as part of microbial denitrification which occurs in microorganisms that are able to respire using alternative electron acceptors during anaerobic conditions (Rütting et al., 2011, Jetten, 2008). The prominence of NO means dedicated systems for detoxification are required in order for *Salmonella* to survive. The following detoxification systems present in *Salmonella* will be discussed in this chapter. NO detoxification occurs during *Salmonella* infection in order for cells to remain viable in macrophages. This is because macrophages provide an extremely stressful environment being anoxic, acidic, and high in ROS and RNS (Aktan, 2004). Therefore, *Salmonella* have a vast number of genes responsible for detoxification and these often have multiple functions due to the succinct nature of bacterial genomes. Regulation of these systems are almost as important as the detoxification itself. They are considered an energetically expensive processes so these genes will only be expressed during exposure to NO or another stimulant (Gilberthorpe et al., 2007).

The flavohaemoglobin HmpA is important as it catalyses two separate reactions, one as a dioxygenase which forms nitrate from NO (Gardner et al., 1998) and the other as a reductase which forms N_2O and H_2O from NO (Rowley et al., 2012). The deletion of *hmp* in *E. coli* results in sensitivity to NO and other RNS. In *Salmonella* the same is seen, but only aerobically with a minimal effect anaerobically. Removal of *hmpA* does attenuate the ability for the strain to survive in macrophages (Gilberthorpe et al., 2007, Karlinsey et al., 2012). Fur primarily has a role in iron homeostasis, but these Fe^{2+} and Zn containing monomers form homodimers where they bind DNA in the promoter region denoted as the iron box. This region is a conserved 19 bp sequence that is found upstream of multiple iron controlling genes including but not limited to *sod*, *suf* operons and *fur* itself (Dubrac and Touati, 2000, Escolar et al., 1997). The process of binding represses these genes and although *hmpA* is indirectly impacted by Fur, the flavohaemoglobin does not have an iron box upstream of the gene (Justino et al., 2005). The deletion of *fur* results in increased intracellular iron which can be lethal by accumulation of

DNA damaging ROS (Touati et al., 1995). In *E. coli* the methionine synthesis regulator MetR has been linked to positively regulate *hmp*. It indirectly does this through upregulating MetR to compensate for NO dependent loss of homocysteine and *hmp* is expressed to counteract the NO which protects the bacteria (Membrillo-Hernández et al., 1998). Although, this has not yet been proven to occur in *Salmonella*.

NorV and NorW are tetrameric proteins and form redox partners which generates NO reductase activity through creating an electron transport chain. NorV contains a zinc-β-lactamase like domain at the N-terminus whilst also containing non-haem di-iron site. There is also a flavodoxin-like domain and rubredoxin-like centre at the C-terminus, hence its name flavorubredoxin (Gomes et al., 2000). The *norV* gene is expressed upon NO and H_2O_2 exposure. Authors proposed that H_2O_2 interferes with NO binding to NorR iron centres thereby inhibiting NO reductase activity (Baptista et al., 2012). Deletion of *norV* gives no specific phenotype for intracellular survival in macrophages or during infection of mice (Mills et al., 2008). The activator NorR also has three crucial domains that enable the transcriptional regulator to function. These include the C-terminal DNA binding domain, σ^{54} dependent activator region with ATPase activity that is dependent of NO binding to the N-terminal mononuclear iron cluster. The deletion of *norR* inhibits the endogenous production of NO through DNRA (D'Autréaux et al., 2005).

The solely anaerobic NrfA periplasm located protein is known to form a complex with NrfB which is required for enzyme function. These dimers are also redox partners with pentahaem cytochromes (Clarke et al., 2007, Bamford et al., 2002). Similarly, the deletion of this gene singularly does not give a distinct phenotype from the parental strain (Rowley et al., 2012), but a double knockout mutant without *nrfA* and *norV* results in increased sensitivity to endogenously produced NO (Mills et al., 2008).

The genes that are highly upregulated during nitrosative stress include the NO detoxification genes *hmpA*, *norV* and *ytfE*, which is proposed to have a role in iron-sulphur cluster repair. This gene is negatively regulated by FNR and positively induced by Fur (Justino et al., 2005). Mukhopadhyay et al. (2004) documented that YtfE has regions of homology with NO regulators which could indicate other regulatory roles for this protein. The crystal structure was resolved by Lo et al. (2016) and the authors proposed that YtfE serves as a NO scavenger after nitrosative stress which serves to help N_2O production in low concentrations of NO. The protein has orthologues in many other species supporting the important role of YtfE. It has a di-iron core which is likely coordinated by histidine and carboxylate residues. Balasiny et al. (2018) demonstrated that YtfE reversibly released NO from nitrosylated Fe-S centres which

could be done directly or through NO attached to the nitrosylated iron atom which would result in an iron-deficient enzyme that could be repaired.

Originally, Hcp was considered a hydroxylamine reductase, but authors suggested this is not the primary substrate of the enzyme. The fact that it is regulated by NsrR and becomes upregulated during NO exposure strongly suggests that Hcp has an unidentified role in protecting bacteria during nitrosative stress (Vine and Cole, 2011). Studies suggest that Hcp is a high affinity NO reductase present in anaerobic bacteria which is dependent on iron clusters to perform S-nitrosylase activity (Seth et al., 2018). Hcr was determined as the natural redox partner for Hcp because *hcr* encodes an NADH reductase containing a FAD and 2Fe-2S cluster (van den Berg et al., 2000). Reactions performed by Hcp remained elusive until recently, but it is known to catalyse NO to N_2O whilst interchangeably being oxidised to reduced Hcp. Moreover, the reduced Hcp is more likely to bind the N_2O product and dissociation occurs when the hybrid cluster is re-reduced (Hagen, 2019).

YgbA is considered a conserved hypothetical protein with an unknown function, but it has been investigated in NO detoxification because it is regulated by NsrR. Notably, *ygbA* is upregulated upon lower levels of NO exposure in comparison to other NsrR regulated genes involved in NO detoxification (Karlinsey et al., 2012). Furthermore, high upregulation of *ygbA* occurred in the presence of NO donors and raised mRNA levels continued for around 90 minutes after exposure proving that YgbA plays a role in the nitrosative stress response (Mukhopadhyay et al., 2004). One study found that *ygbA* was among other top ranked genes in the SPI-1 interactome suggesting a role for YgbA in invasion into non-phagocytic cells (Lahiri et al., 2014). Previous researchers have attempted to generate a *ygbA* deletion mutant but were unsuccessful and this could be due to the method of mutation generation (Gilberthorpe et al., 2007). YgbA belongs to the ygbA NO protein family domain which characteristically contains a group of evolutionarily related proteins with a promoter that is inducible by N_2O. This gene is known to predate the insertion of the *avrA-invH* TTSS in the *Salmonella* ancestor which are vital for pathogenicity and supports the importance of *ygbA* (Lerminiaux et al., 2020).

As previously introduced, NsrR is an important transcriptional repressor that controls expression of numerous NO detoxification genes and putative tellurite resistance genes. It becomes deactivated during bacterial exposure to NO through the NO sensitive Fe-S cluster. In addition to *nrfA* and *hmpA*, NsrR also controls *ytfE*, *ygbA*, *hcp-hcr*, *yeaR-yoaG*, *STM1808* and *tehB* (Karlinsey et al., 2012). NsrR has been investigated in *E. coli* and researchers found that selenate/tellurate reduction systems encoded on *ynfEF* and *moeA* were also controlled

by this transcriptional regulator (Fujita et al., 2020). In fact, they discovered that *moeA* was positively regulated by NsrR; thus, proving other functions for this transcription factor.

The other major transcription factor is FNR which is extremely important for anaerobic growth particularly changing gene expression during the transition from aerobic and anaerobic respiration. Similar to NsrR, it also contains an Fe-S cluster, and this is typically 4Fe-4S which becomes transformed to 2Fe-2S in the presence of oxygen which prevent DNA binding (Khoroshilova et al., 1997). Some genes become activated by FNR including *napA*, *nrfA*, *nirB* and *hcp*. While *hmpA*, *ytfE*, *ygbA*, *cyoA* and *yeaR-yoaG* are repressed by FNR (Constantinidou et al., 2006). Interestingly, the presence of NO also impacts the 4Fe-4S cluster resulting in a dinitrosyl-iron-cysteine complex consequently preventing dimerization of the transcriptional regulator and preventing DNA binding. In deletion mutants, anaerobic growth is severely attenuated (Gilberthorpe and Poole, 2008).

The Rowley laboratory was able to successfully generate *ΔygbA* to allow for phenotypic investigation. There is currently a gap in the literature surrounding the role that YgbA plays in nitrosative stress and what phenotypes are present in a deletion mutant. Especially how this reflects in macrophage survival. Other things that could be investigated include the effects of tellurite on the identified NO detoxification systems which could enhance our understanding of how the nitrosative stress response and tellurite resistance mechanisms interact. This chapter will focus on *hmpA*, *ytfE*, *hcp* and *ygbA* in the context of aerobic and anaerobic exposure to tellurite and NO. Finally, assessing the deletion of *ygbA* and how this impacts *Salmonella* to invade and replicate within macrophages.

5.2. Chapter aims

This chapter aims to prove the correlation between nitric oxide detoxification and tellurite resistance by showing NO detoxification deletion mutants have sensitivity to tellurite. As previously identified, *ΔytfE* experienced growth defects anaerobically during higher concentrations of tellurite exposure but not aerobically (Johnston, 2017). This could be hypothesised to occur here, but lower concentrations were being tested so the effect may not be as prominent. Hcp has not been tested in the presence of tellurite but its redox pair Hcr is an NADH reductase so deletion of *hcp* could inadvertently impact the function of Hcr. Using these assumptions, the addition of tellurite would negatively impact *Δhcp*. The phenotype elicited in *ΔhmpA* during NO exposure is noted in the literature (Gilberthorpe et al., 2007) and predicted for this study. In terms of tellurite exposure, this has not been tested but the importance of HmpA in NO detoxification means it can be predicted that tellurite will reduce growth of *ΔhmpA*. The opposite can be said for *ΔnsrR* as deletion of this repressor allows almost free expression of the NO detoxification and tellurite resistance genes. Therefore,

growth of *ΔnsrR* will display little to no effect in the presence of NO and tellurite. Previous research has identified that *ygbA* is upregulated upon NO exposure; thus, it can be hypothesised that the deletion of *ygbA* will result in deficient growth during NO exposure and this could also reflect in reduced survival rates in macrophages.

This chapter aims to:

1. investigate the phenotypes present in deletion mutants of Hcp, YtfE, YgbA, HmpA and NsrR in response to tellurite in low and high concentration in the presence and absence of oxygen.
2. Assess how the deletion of *ygbA* in SL1344 impacts growth during aerobic and anaerobic NO exposure and how this compares to WT, *ΔhmpA* and *ΔnsrR*.
3. Characterise the *ΔygbA* strain during macrophage infection compared to WT.

These aims were achieved by:

- Conducting growth curves during tellurite (0.3 μM) exposure and deta NONOate (5 mM) in the presence of and without oxygen.
- Minimum inhibitory concentration assays and percentage survival assays which were done aerobically with various concentrations of tellurite (0.1 – 5 μM).
- Gentamicin protection assays which involved infecting RAW 264.7 macrophages with WT SL1344 and *ΔygbA* and Cfu/mL values were used to gauge each strain's ability to survive.

5.3. Results

5.3.1. Strains display similar growth aerobically and anaerobically

Similarly, proof was required that deletion of NO detoxification genes *hmpA*, *ytfE*, *hcp*, *ygbA* and regulator *nsrR* generated in SL1344 were not harmful to growth in non-limiting conditions. This was found to be true during aerobic and anaerobic conditions in LB. This proves any phenotypes identified are due to the lack of the particular proteins being studied. There were no significant differences between growth rates of these strains aerobically (p=0.1, F_5=2.28) or anaerobically (p=0.3, F_5=1.29).

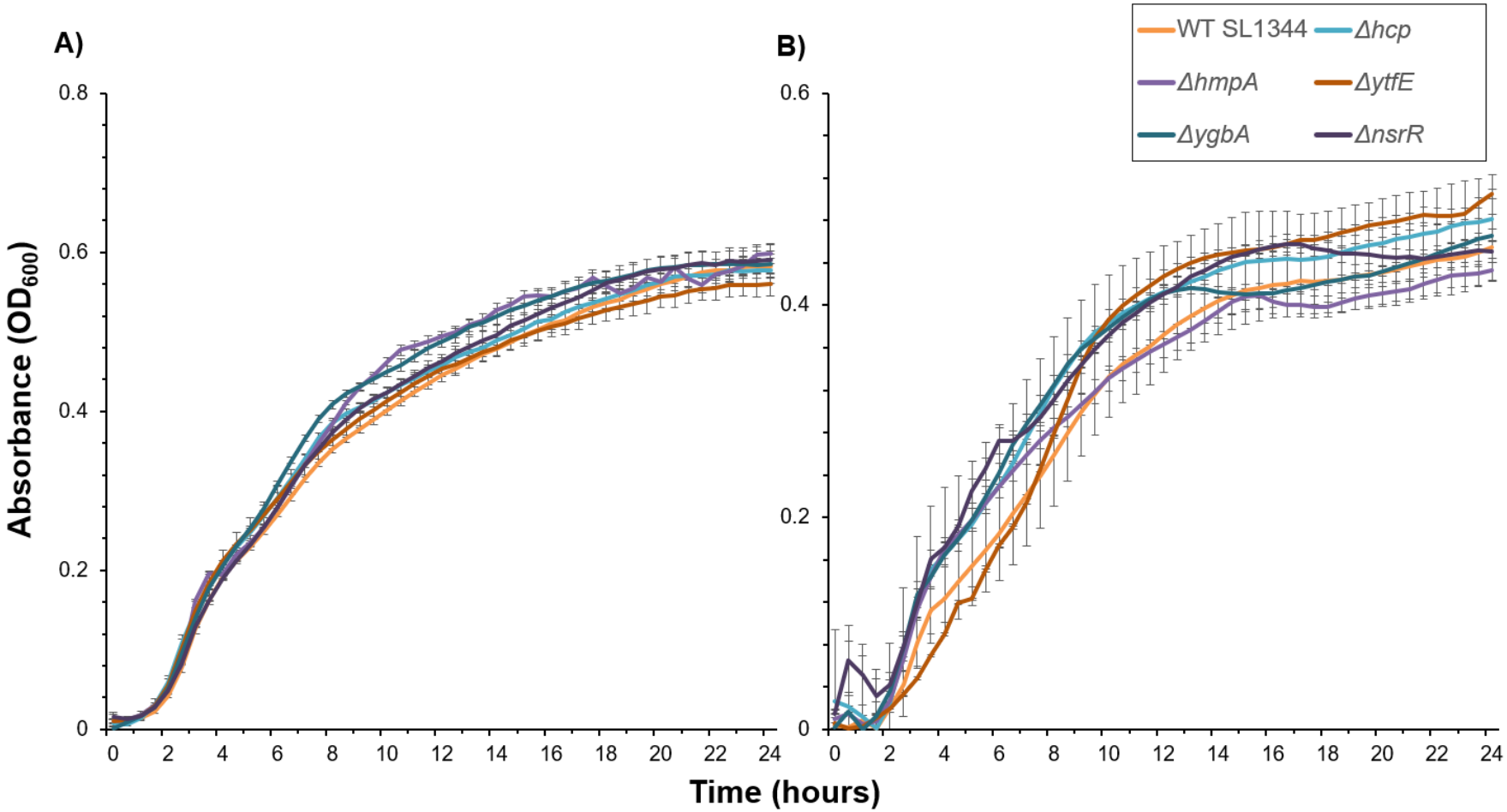


Figure 32. Strains lacking in nitric oxide detoxification genes grow similarly to WT aerobically and anaerobically.
Cultures were grown in 1 mL LB in a 24-well plate and grown over 24 hours with 30-minute interval readings of optical density taken at 600 nm. Data is comprised of three biological replicates with standard error bars plotted for each strain.

5.3.2. HmpA is required for aerobic protection and YtfE is important for the anaerobic protection against tellurite

The strains were subjected to 0.3 μM tellurite to identify any phenotypes in the presence and absence of oxygen.

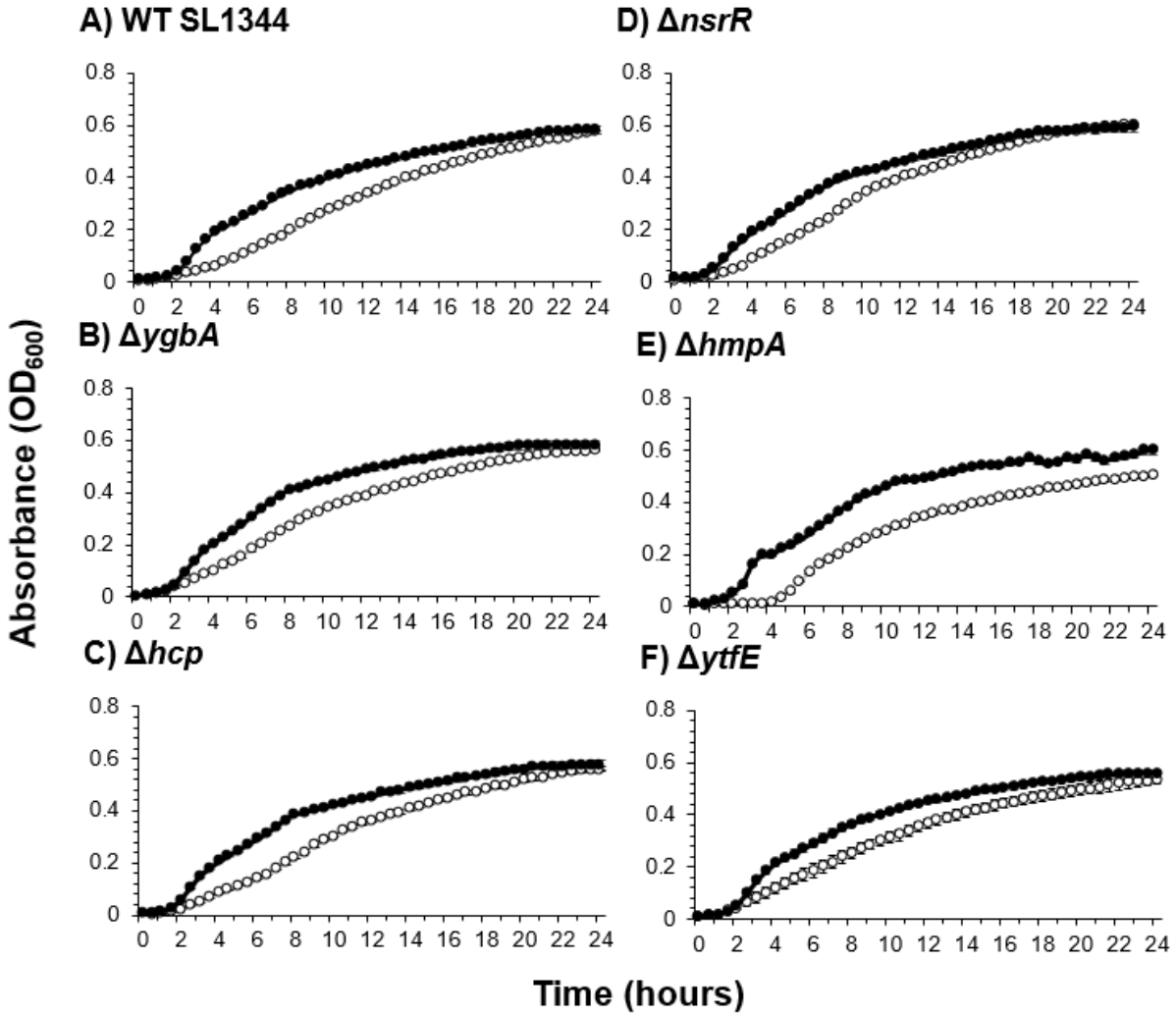


Figure 33. The flavohaemoglobin HmpA is important for aerobic protection against tellurite. Strains were growth with the concentration of 0.3 μM potassium tellurite (white circles) or without (black circles) in aerobic conditions in 1 mL LB. Readings of optical density at 600 nm were taken every 30 minutes over 24 hours. A) WT, B) *ΔygbA*, C) *Δhcp*, D) *ΔnsrR*, E) *ΔhmpA*, F) *ΔytfE*. Data shows three biological repeats and standard error is plotted for each strain.

The effects of tellurite on WT have already been discussed in Chapter 3 and features here as a comparison for the NO detoxification mutants. It appears that the lack of YgbA and Hcp does not impact growth any more than is observed in WT as both have a reduction in growth rate of 48% and 50%, respectively. The same cannot be seen in *ΔnsrR* where tellurite has less of an impact (42% reduction in growth rate) as the deletion of the repressor can allow upregulation of tellurite resistance proteins. A similar pattern of 43% loss in growth rate can

be observed for *ΔytfE* proving that this protein must not play much of a role in tellurite resistance aerobically. Conversely, deletion of the flavohaemoglobin results in an extended lag phase during tellurite exposure. This could highlight the importance of this NO detoxification protein in tellurite detoxification. The effects of tellurite were assessed anaerobically to investigate if a similar phenotype was seen in the mutants as aerobic exposure.

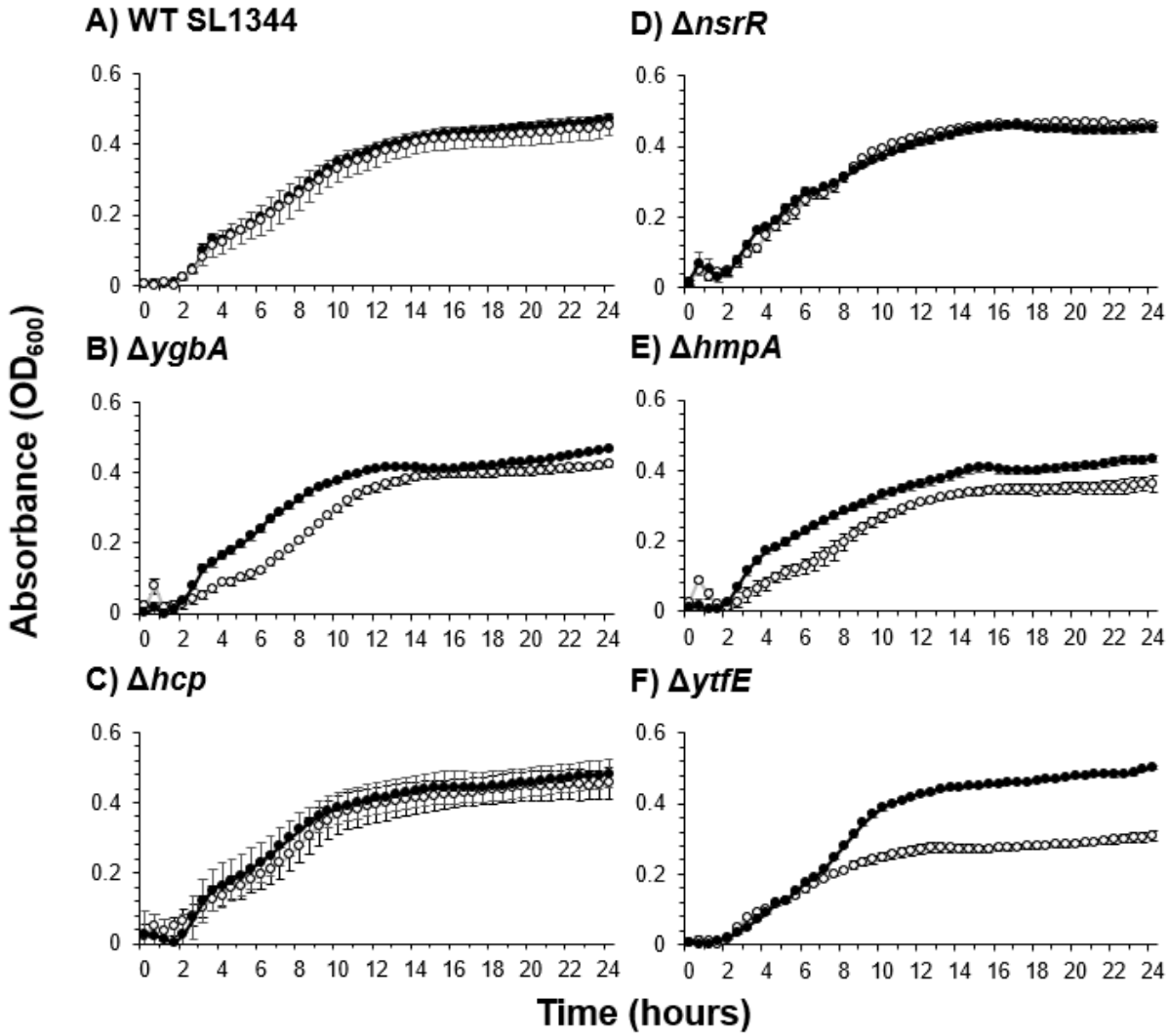


Figure 34. Tellurite impacts growth of *ΔytfE* and to a lesser extent *ΔygbA* in anaerobic conditions.
Strains were growth with the concentration of 0.3 μM potassium tellurite (white circles) or without (black circles) in anaerobic conditions in 1 mL LB. Readings of optical density at 600 nm were taken every 30 minutes over 24 hours. A) WT, B) *ΔygbA*, C) *Δhcp*, D) *ΔnsrR*, E) *ΔhmpA*, F) *ΔytfE*. Data shows three biological repeats and standard error is plotted for each strain.

The WT SL1344 did not suffer with the addition of the same concentration of tellurite compared to aerobic conditions. In comparison to aerobic phenotypes, lesser harmful effects of tellurite were detected through growth change in the *Δhcp* (3% reduction in growth rate), *ΔhmpA* (6%

reduction) and *ΔnsrR* (26% gain in growth) occurred anaerobically. The deletion of YtfE resulted in 47% reduction in growth rate and to a lesser extent 8% loss of growth rate in *ΔygbA* which impacted *Salmonella* ability to grow in 0.3 μM tellurite anaerobically.

As tellurite appeared to have differential effects at such low concentrations, other concentrations were assessed using percentage survival and MIC assays. At concentrations of 0.1 μM – 0.5 μM K_2TeO_3 *Δhcp* and *ΔhmpA* did not experience significant differences in survival compared to WT. Survival of *ΔygbA* was variable and tended to be higher in these lower concentrations of tellurite. Whereas, between 1 μM – 5 μM survival in this strain was significantly lower than WT suggested a role for this protein against tellurite resistance. Similarly, *ΔytfE* survival appeared similar to WT apart from at 0.1 μM and 5 μM where differences were more pronounced, and this could implicate YtfE in tellurite protection. Results support that deletion of NsrR causes higher levels of survival in the presence of tellurite compared to WT. Differences between the two could be linked to the method of growth measurement and how cells were exposed to tellurite. Results in MICs were 12-hour liquid grown aerobic cultures, but isolated colonies were grown on plates containing tellurite or normal LB for percentage survival assays.

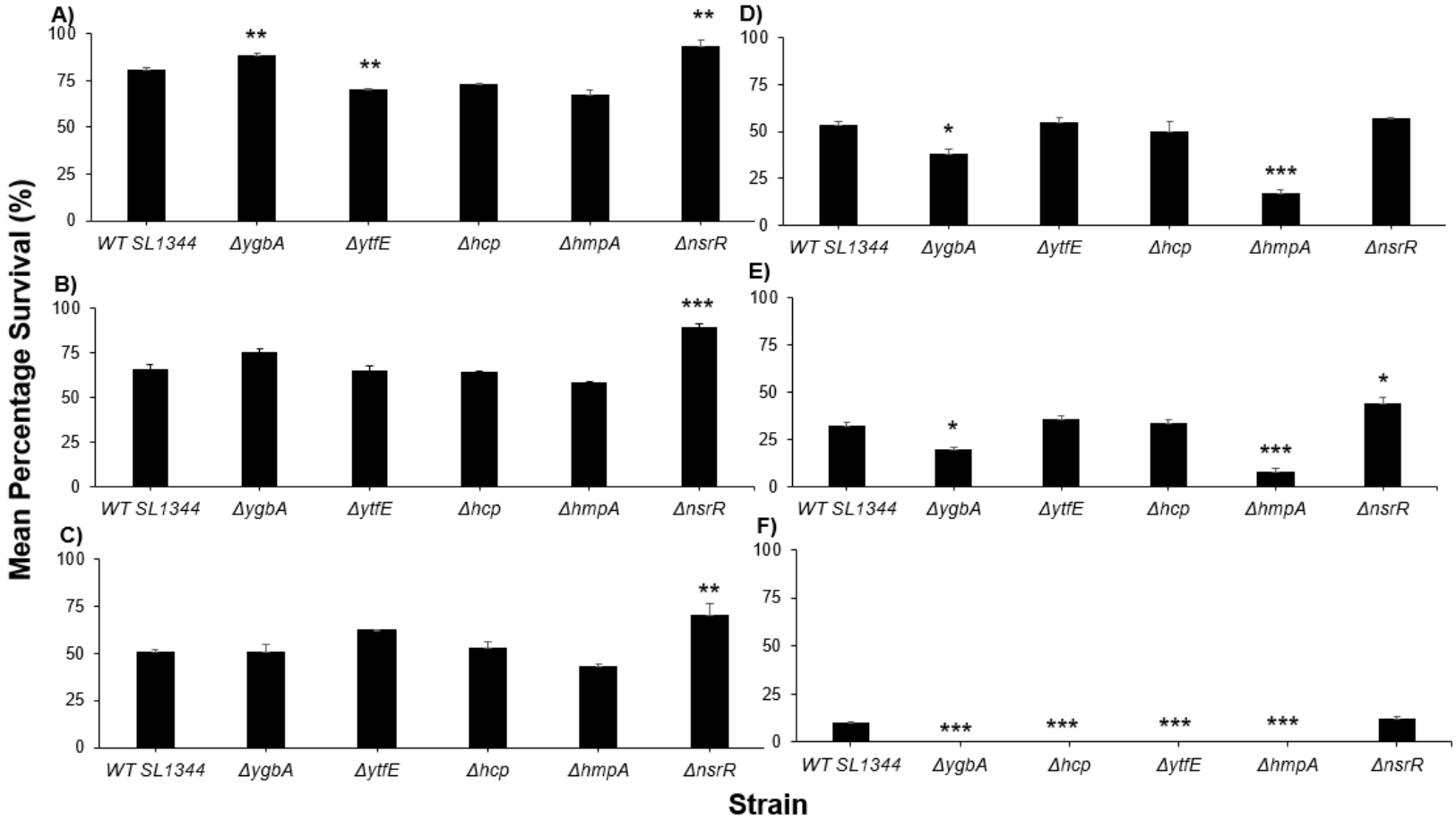
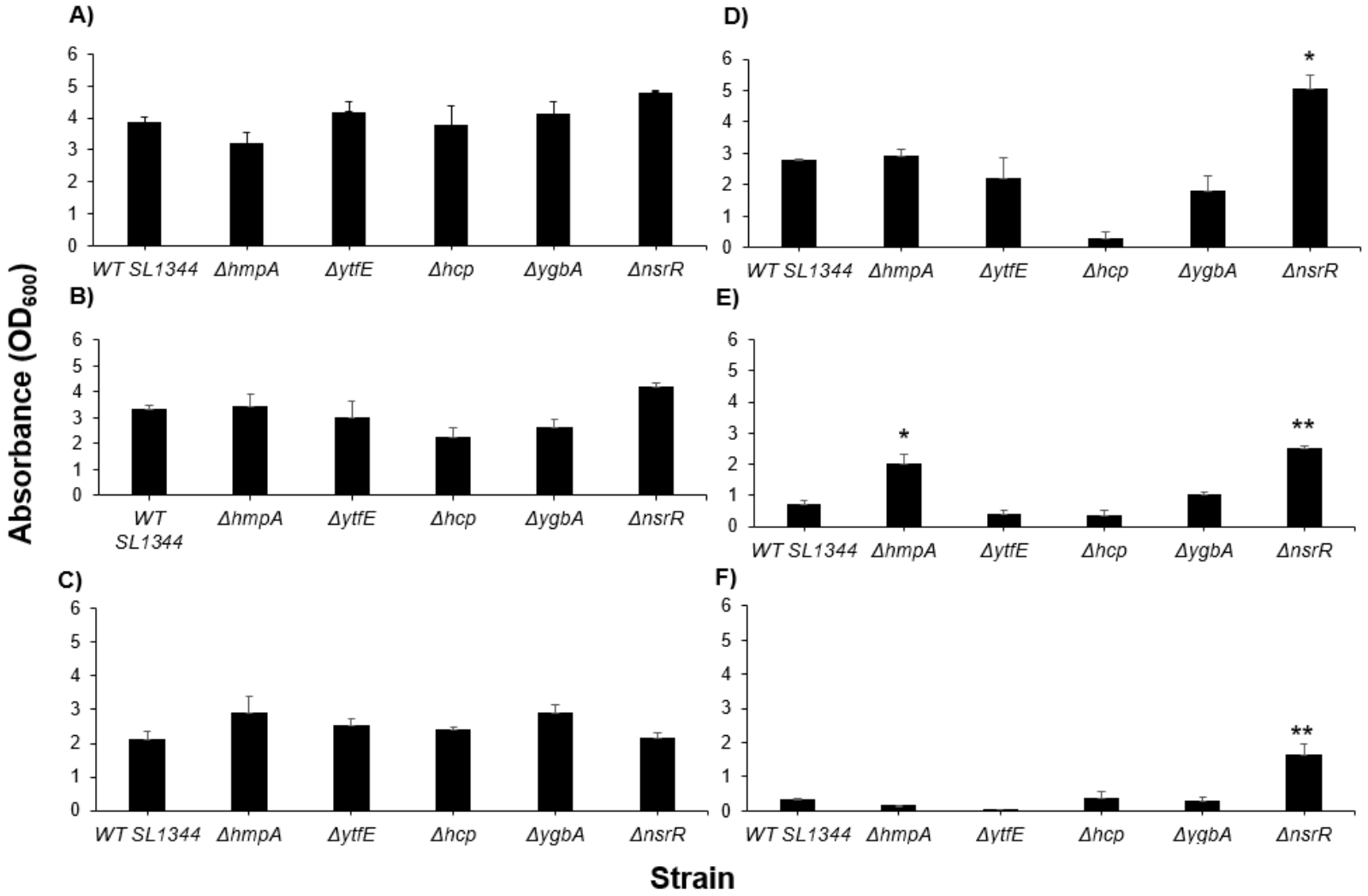


Figure 35. The effects of tellurite at 5 μM are significantly deleterious in mutants lacking in YgbA, Hcp, YtfE and HmpA.
Percentage survival from tellurite was calculated using Cfu/mL values on control plates (no K_2TeO_3) with Cfu/mL values containing 0.1 μM (A), 0.2 μM (B), 0.5 μM (C), 1 μM (D), 2 μM (E), 5 μM (F) K_2TeO_3. Data is composed of three biological repeats and standard error is displayed on bars. Statistical analysis was conducted using ANOVA single factor with replication and post hoc Tukey's Test for multiple comparisons. Asterisk (*) indicates significance: * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.



*10***Figure 36.** Deletion of NsrR caused increased growth compared to WT in higher concentrations of tellurite.
Strains were grown for a period of 24 hours with no tellurite (A), 0.2 μM (B), 0.5 μM (C), 1 μM (D), 2 μM (E) and 5 μM (F). Data is composed of three technical repeats, standard error has been plotted and statistical analysis conducted using ANOVA single factor with replication and post hoc Tukey's Test with multiple comparisons. Asterisk (*) indicates significance: * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

MIC values gave a different perspective on aerobic growth observed after 12 hours of incubation with tellurite. There were no significant differences in OD_{600} values without the presence of the oxyanion in rich LB. Patterns did emerge when strains were exposed to 1 μM K_2TeO_3 which highlights that *Δhcp* suffers greatly during liquid culture of the compound which is similar for *ΔygbA*. The same cannot be said for *ΔhmpA* which had similar or higher OD_{600} levels to WT with the exception of 5 μM that diminished growth substantially for many of the strains, including *ΔytfE* that was most greatly impacted. This was particularly interesting as lower concentrations of tellurite resulted in similar levels of growth in WT and *ΔytfE*.

5.3.3. Nitric oxide has a limited effect on mutants lacking in YgbA *in vitro*

As the genes controlled by NsrR have been highlighted for NO detoxification this was further investigated for YgbA as there is limited data in the literature. Therefore, growth rates were calculated using the natural log of OD_{600} values to allow differences to be compared when deta NONOate was incubated with strains. WT was used to compare reactions to exposure to NO whereas *ΔhmpA* was used as a negative control and *ΔnsrR* as the positive control. This is because other studies have identified that increased growth occurs in ΔnsrR, but an extreme reduction in growth is seen in *ΔhmpA* (Gilberthorpe et al., 2007).

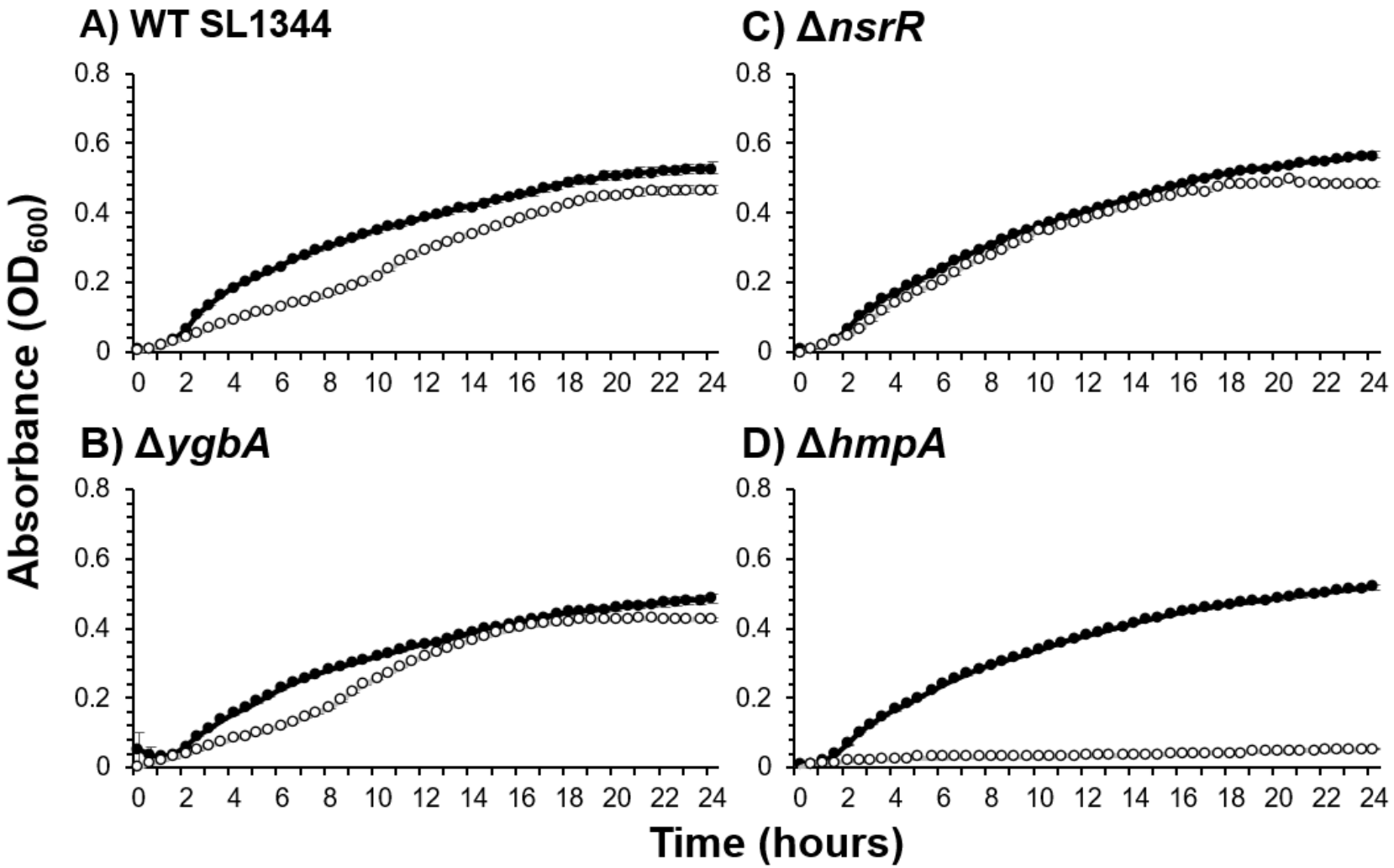


Figure 37. HmpA is important for aerobic protection against NO but YgbA is not essential.
Strains were grown in 1 mL of LB with (white circles) and without (black circles) 5 mM deta NONOate in a 24-well plate and automated optical density readings at 600 nm were taken every 30 minutes over a period of 24 hours in aerobic conditions. A) WT, B) *ΔygbA*, C) *ΔnsrR*, D) *ΔhmpA*. Data is composed on three biological repeats and standard error is plotted on bars.

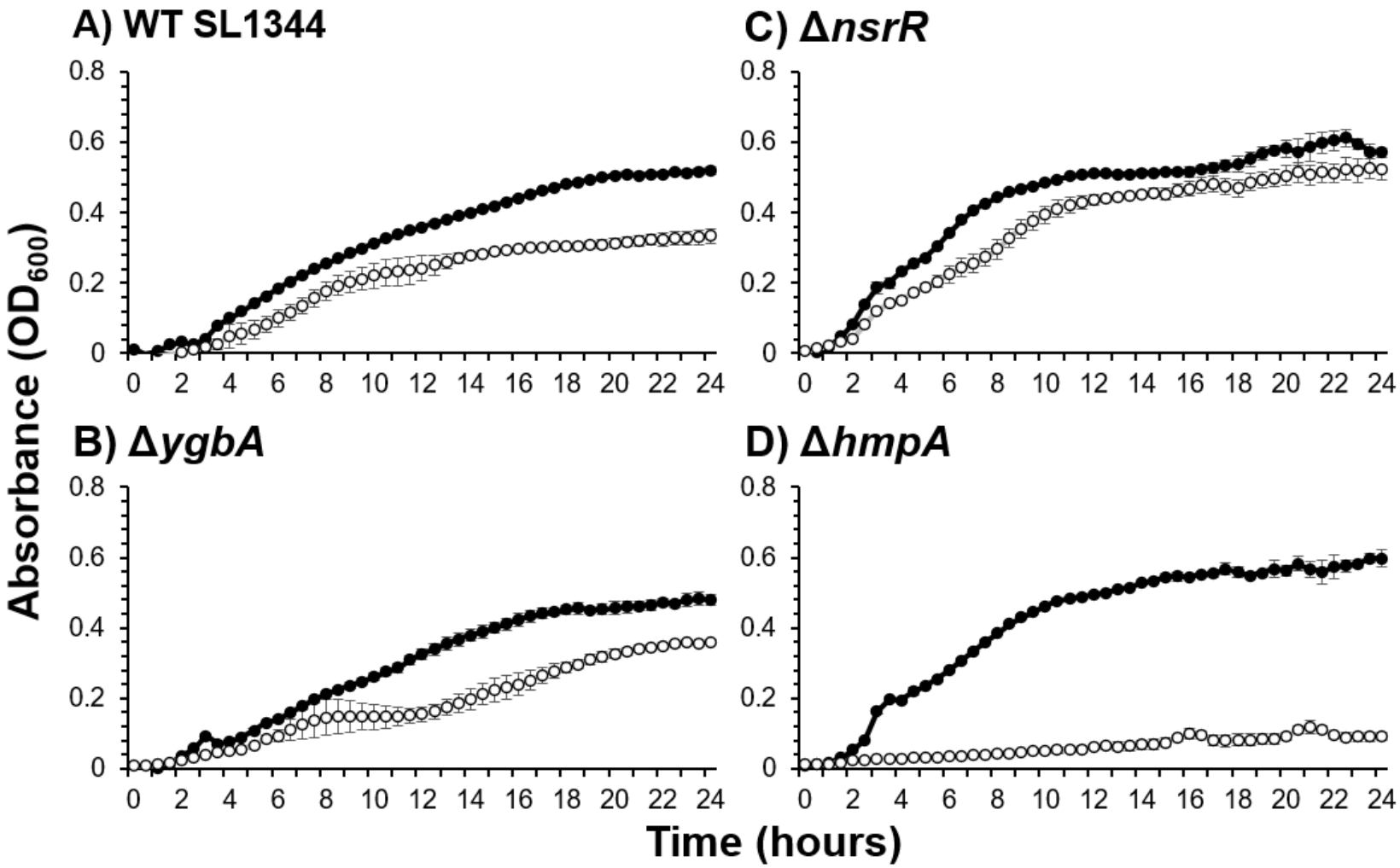


Figure 38. Lack of oxygen enhanced the detrimental effects of NO in all strains.
Strains were grown in 1 mL of LB with (white circles) and without (black circles) 5 mM deta NONOate in a 24-well plate and automated optical density readings at 600 nm were taken every 30 minutes over a period of 24 hours in anaerobic conditions. A) WT, B) *ΔygbA*, C) *ΔnsrR*, D) *ΔhmpA*. Data is composed on three biological repeats and standard error is plotted on bars.

As previously mentioned, WT experiences a growth loss of 30% aerobically and 17% anaerobically (chapter 3). The *ΔnsrR* strain conversely has an increased growth rate by 5% during aerobic exposure to NO. However, this becomes a loss of 17% anaerobically meaning the deletion of this protein had a greater impact in the absence of oxygen. The *nsrR* mutant had less growth with NO anaerobically as there are other anaerobic NO detoxification systems in place NrfA and NorV which are regulated by other proteins. Alternatively, *ΔhmpA* had greater reductions of 96% and 92% in aerobic and anaerobic growth, respectively. Growth of *ΔygbA* was reduced by 9% during aerobic conditions but anaerobically this was further reduced by 35% indicating that YgbA could have a role in anaerobic NO protection.

5.3.4. YgbA is important for *Salmonella* survival in macrophages

Information learned about YgbA from *in vitro* NO exposure growth assays justified further investigation to gauge the gene's importance during macrophage infection. RAW 264.7 murine macrophages were cultured in DMEM media without any cytokine treatment. Once a sufficient confluence was obtained, cells were split and seeded in 12 well plates to generate a 1:10 multiplicity of infection. Assessing the level of bacterial cells recovered after 2 hours allows the comparison of how *Salmonella* invade the macrophages. Whereas 24 hours of incubation allows the comparison of ability to replicate within the macrophages.

The deletion of YgbA resulted in significantly reduced bacteria cell recovery from lysed macrophages. Thus, indicating that *ΔygbA* is less capable of invading and surviving within murine macrophages than WT SL1344. The deficiency would predictably be larger in cytokine activated macrophages. This data supports that YgbA may have a role in NO detoxification, but as there are many stressors present in macrophages, including ROS, low pH, lack of oxygen. Therefore, further investigation is needed to prove that the production of NO was the reason for this attenuation.

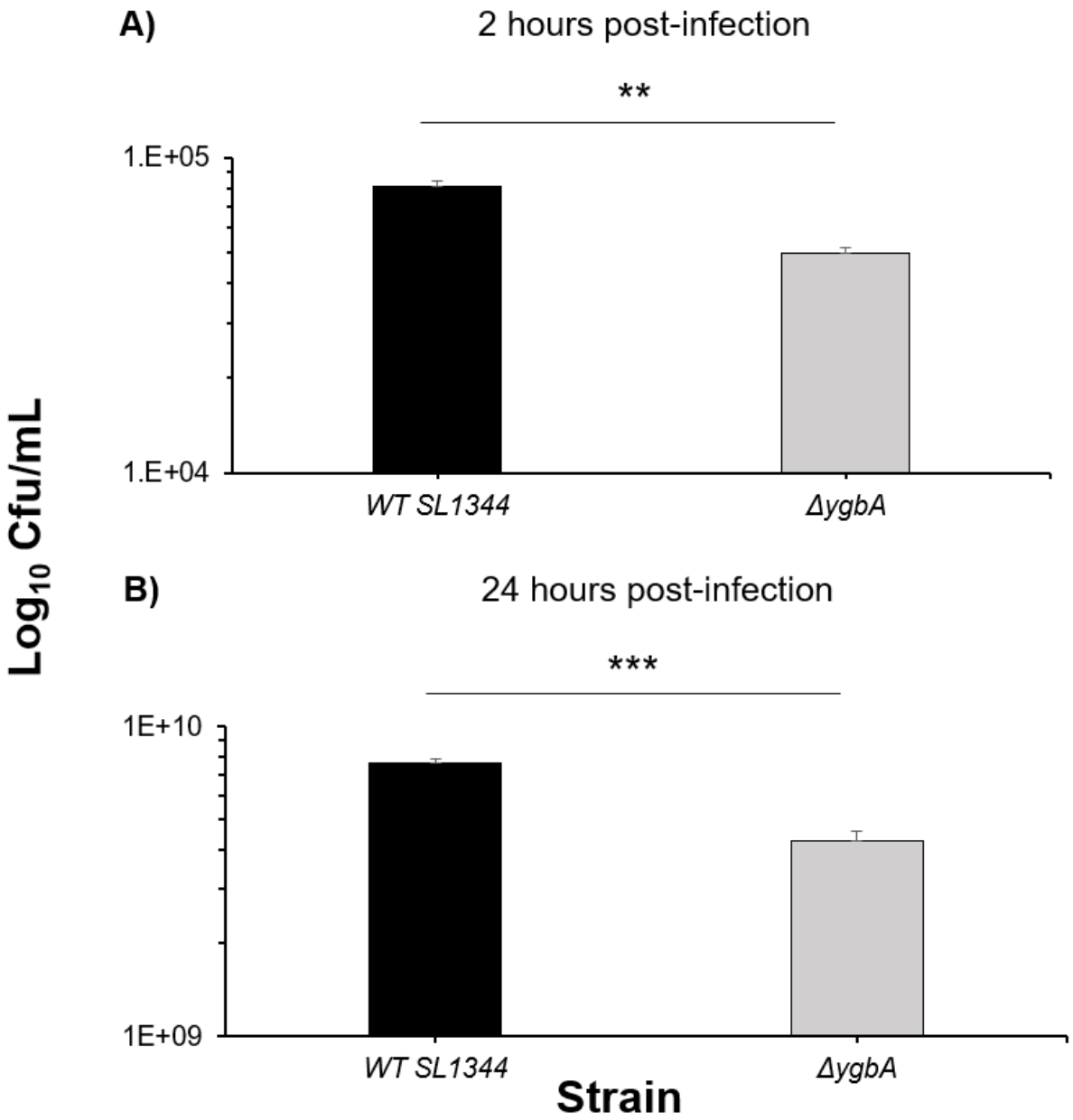


Figure 39. Deletion of YgbA results in attenuated survival within murine macrophages. Untreated RAW 264.7 macrophages were infected with WT SL1344 and *ΔygbA* in a multiplicity of infection of 10:1 and incubated for 2 hours (A) and 24 hours (B). Macrophages were lysed with 1% Triton X-100, and the cell lysate was serially diluted to allow Cfu/mL calculations and the log_{10} was plotted for each strain. The data was composed of three biological repeats with SEM plotted and normalised to a dose of 10^6 *Salmonella*. Statistically compared using a two-tailed t-test to compare Cfu/mL at 2 hours (T_3=8.02 , p=0.004) and 24 hours (T_4=8.19, p=0.001). Asterisk denotes significance (**) p<0.01 and (***) p<0.001.

5.4. Discussion

The purpose of this chapter was to investigate the roles that Hcp, YtfE, YgbA, HmpA and NsrR play during tellurite exposure which may have an overlapped function with NO detoxification. If deletion mutants demonstrated lack of growth because of the oxyanion, it could signify that the protein gives a level of tellurite resistance in WT SL1344. This may not be their primary function, as expressed earlier, *Salmonella* is unlikely to naturally come into contact with tellurite. The toxicity of NO and other RNS are responsible for the repertoire of NO detoxification systems present in *Salmonella*. Various studies before having been completed to confirm phenotypes to NO for Hcp and YtfE, they were not investigated here, and focus was drawn to YgbA as there is a lack of research on protein function in NO detoxification in *Salmonella*. As HmpA is crucial for NO detoxification a deletion mutant displays a severe growth deficiency in the presence of NO donors and the opposite is seen in *ΔnsrR* which displays little impact to growth (Gilberthorpe et al., 2007). Therefore, *ΔhmpA* and *ΔnsrR* were used as negative and positive controls, respectively which could aid phenotypic investigation of *ΔygbA*. YgbA was also investigated for importance in *Salmonella* survival in macrophages.

To investigate the impact of tellurite on *Δhcp*, *ΔytfE*, *ΔygbA*, *ΔnsrR* and *ΔhmpA*, strains were grown aerobically and anaerobically in LB and in the presence of different concentrations of tellurite. It appeared that 0.3 μM K_2TeO_3 was the minimum concentration required to observe differences between WT SL1344 and deletion strains. Growth of deletion strains were unaffected in LB without tellurite. This allowed the comparison of growth rates and to ensure that absence of such proteins did not impact the fitness of the strains. Therefore, a decrease in growth of all strains upon addition of K_2TeO_3 was predicted. This was not observed for *Δhcp* but growth differences were observed among *ΔygbA* and *ΔytfE* compared to WT. Other studies have suggested Hcp to have oxidoreductase activity (van den Berg et al., 2000) which can be useful for tellurite reduction but may also rely on the redox partner Hcr, which would impact growth of *Δhcp* but this was not seen.

The reduction in growth seen in *ΔhmpA* was more prominent aerobically compared to other strains in growth curves which may indicate that HmpA gives a level of aerobic protection against tellurite. The defect in growth was less prominent on *ΔhmpA* anaerobically; however, the notable decrease in *ΔytfE* highlights that YtfE confers anaerobic (not aerobic) resistance to tellurite which supports previous work (Johnston, 2017). The flavohaemoglobin functions as a nitric oxide reductase and some of these enzymes display the ability to reduce tellurite to tellurium which has been noted in terminal oxidases responsible for denitrification in gram negative bacteria (Trutko et al., 2000). Furthermore, research by Avazéri et al. (1997) suggested the use of nitrate reductases in *E. coli* for tellurite reduction. Other enzymes which

contain tellurite reducing properties include type II NADH dehydrogenase, dihydrolipoamide dehydrogenase and characteristics of flavoproteins including catalytic cysteine residues and FAD/NAD(P)$^+$ binding domains (Arenas-Salinas et al., 2016). YtfE has been proven to use NADH as an electron donor to repair iron-sulphur clusters which become damaged through oxidative and nitrosative stress (Vine et al., 2010). Perhaps YtfE reduces damage to Fe-S clusters during tellurite exposure or may even function in reduction through sacrificing NAD(P) or NAD(P)H oxidation which was supported by a previous study conducted by Calderón et al. (2006). But this activity only occurs without oxygen, as *ytfE* is repressed by FNR.

Notably, *ΔnsrR* experienced the lowest reduction in growth rate upon addition of tellurite as the lack of the repressor allows expression of tellurite resistance genes *STM1808*, *yeaR* and *tehB*. In fact, *ΔnsrR* experiences sensitivity to oxidative stress and reduced survival within macrophages which highlights that controlling such genes is imperative during *Salmonella* infection (Gilberthorpe et al., 2007). Having regulators such as NsrR, allow expression of relevant detoxification genes when required which conserves energy during infection. Upregulated expression will not solely occur in *ΔnsrR* as other detoxification and resistance genes are controlled by other regulators.

The effects of tellurite which have been previously outlined, stimulate oxidative stress in bacteria and higher concentrations of this oxyanion was predicted to be more deleterious for deletion strains. The concentrations used in the growth curves may be too low to observe the effects of tellurite on other NO detoxification genes. Therefore, further assays took place to assess percentage survival and OD_{600} after growth with much higher concentrations of tellurite. This does occur for *ΔygbA* and *ΔhmpA* which appear to have significantly reduced survival from 1 μM K_2TeO_3. Whereas *ΔytfE* and *Δhcp* have similar survival rates to WT until 5 μM where growth was entirely depleted in these mutants. This does support that HmpA is important for aerobic protection and YtfE for anaerobic protection as these assays were done aerobically. Interestingly, the deletion of *nsrR* does protect *Salmonella* from tellurite, growth was similar to WT in lower concentrations, only becoming significantly increased above 1-2 μM K_2TeO_3 which differs slightly between OD_{600} and survival readings.

As the function of YgbA remains unresolved, the phenotypic study of *ΔygbA* provided some insight into possible roles for tellurite resistance. Aerobically, YgbA did not appear to have any role in protection against tellurite, but anaerobically, the addition of tellurite was more attenuated compared to WT. Therefore, indicating that this hypothetical protein contributes to tellurite resistance even if this is not the proteins primary function. The knowledge that *ygbA* is upregulated in the presence of NO (Mukhopadhyay et al., 2004) stimulated phenotypic analysis on *ΔygbA* in the presence of 5 mM deta NONOate. Similar to Karlinsey et al. (2012),

YgbA was identified as important for nitrosative stress response particularly for anaerobic protection. Constantinidou et al. (2006) proposed YgbA had a role in aerobic nitrosative stress because the gene is repressed by FNR which is oxygen sensitive, but this is not supported in the present study.

The lack of growth in *ΔhmpA* with the NO donor is so reduced because the flavohaemoglobin is remarkably important in NO detoxification (Poole, 2020). This phenotype is common and reported by other authors and so was used in this study as a negative control (Gilberthorpe et al., 2007). Whilst *ΔnsrR* was used as a positive control because minimal growth loss occurs which was more prominent without oxygen. Fluctuation in growth can be explained by other regulators controlling detoxification genes such as FNR, Fur, MetR and NorR.

Considering that YgbA was only found to be crucial for anaerobic nitrosative stress elicited with 5 mM deta NONOate in this study, further investigation occurred to assess *ΔygbA* survival in macrophages compared to WT SL1344. This was done to resolve the importance of YgbA in NO detoxification. Significantly lower Cfu/mL *ΔygbA* were recovered after 2 and 24 hours of macrophage infection compared to WT. This suggests that lack of YgbA attenuates *Salmonella* invasion and survival within murine macrophages. A previous investigation conducted by Lerminiaux et al. (2020) found that expression of *ygbA* and *sitABCD* was upregulated inside macrophage vacuoles. Thereby, supporting the critical role identified here for YgbA during macrophage infection.

Further work should be done by conducting anaerobic MIC and percentage survival assays for tellurite exposure to see if that supports the data obtained in growth curves. This could be extended to further assays with deta NONOate or other NO donors to discover if the phenotypes identified are still present. Transcription analysis using qPCR could be performed to understand changes in *ygbA*, *ytfE*, *hcp* and *hmpA* mRNA produced during tellurite exposure. As if they are upregulated by tellurite as well as NO, it would corroborate the link between tellurite resistance and NO detoxification. Gentamicin protection assays could be repeated using IFN-γ activated macrophages which produce more physiologically relevant concentrations of NO. This could be extended to investigate whether oxidative or nitrosative stress is the main protagonist responsible for attenuation in *ΔygbA*. For example, treatment with a NADPH oxidase inhibitor such as Apocynin or an iNOS inhibitor (e.g., Silymarin) would confirm or deny this. Finally, complementation of mutant strains should be conducted to confirm that supplementation of a WT copy of the gene would successfully restore the phenotype observed for tellurite, deta NONOate and macrophage survival.

Chapter 6: General Discussion

As results gained from each chapter have been discussed, this general discussion will focus on how these results fit within the wider literature. Furthermore, assessing what studies can be conducted in future to justify and build on the results gained here.

6.1. Context of study

Salmonella is a globally important enteric pathogen. It causes 98 and 21 millions of cases of gastroenteritis and typhoid fever in humans and causes 155,000 and 200,000 deaths annually, respectively (Majowicz et al., 2010, Marchello et al., 2019). These figures may not even entirely cover the burden experienced worldwide as many milder cases go unreported or misreported as other enteric infections. Not only humans are impacted by this pathogen as *Salmonella* can infect numerous animals and elicit a wide range of diseases as a consequence and allowing zoonotic spread. Predominantly infections begin through consumption of contaminated foods or water and typically children and the elderly are at the highest risk of infection (Hadler et al., 2020). Despite improved sanitation, access to healthcare treatments and preventative vaccinations *Salmonella* sp. remain the leading cause of mortality being prominent in poverty-stricken areas. The enteric disease poses a large economic burden for health systems and NTS infections can even produce further health complications through developing chronic sequelae (Beltran-Fabregat et al., 2006). The requirement of needing time off work can cut productivity by up to 80% which can impact the country's economy (Esan et al., 2020).

As previously emphasised, *Salmonella* is capable of causing gastroenteritis which is typically instigated by generalist NTS (e.g., Typhimurium) or typhoid fever which is caused by host limited serovars (e.g., Typhi). Since the emergence of iNTS, the global burden was estimated at 3.4 million cases with 681,316 deaths reported in 2010 (Crump and Heyderman, 2015, Marks et al., 2017). High risk groups include immunocompromised people such as those that are HIV positive. This has meant outbreaks of iNTS are endemic in regions with high HIV or malaria caseload such as in Africa (Marks et al., 2017). Infections are becoming more complex to treat due to the increase in AMR. Especially in the case of iNTS, which up to 90% of isolates can contain multidrug resistance (MDR) which is alarming (Gordon, 2008). This has meant that there are limited avenues of therapeutics that remain to work against these MDR strains. Furthermore, preventative options are limited as there are only Typhoid vaccinations, and no current vaccines are licensed for use against NTS or iNTS. However, potential avenues for such vaccines are being researched (Tennant et al., 2016). Research has also been targeted towards finding new drug targets and potential vaccination strains to use. For example, the

use of biofilm disrupting drugs, repressing effector proteins or TTSS themselves which would significantly attenuate the virulence of *Salmonella* in hosts (Sandala et al., 2021, Li et al., 2013, Negrea et al., 2007, Askoura and Hegazy, 2020).

Salmonella are remarkably adaptable pathogens as they encounter many different conditions throughout their lifecycle and infection process. This includes, changing temperatures, pH, lack of nutrients, osmotic pressures, reactive oxygen and nitrogen species and oxygen deprivation which can all elicit damage in some form or another (Poole, 2012). Their ability to withstand such changes allow resistance to develop which can enhance their virulence. Being able to survive in rapidly changing environments is partially controlled by stress responses. Of particular interest to this thesis is the nitrosative stress response which allows the detoxification of RNS which are either encountered internally through nitrate/nitrite respiration (Rowley et al., 2012) or externally by macrophages in the host immune response (Eriksson et al., 2003).

6.1.1. The importance of the nitrosative stress response

As *Salmonella* encounter numerous stressors throughout their lifecycle and infection process, they have adapted robust systems to handle such situations. During infection, *Salmonella* utilises SPI-1 for the invasion of enterocytes or M cells to avoid antimicrobial peptides in the gastrointestinal tract (Fàbrega and Vila, 2013). Once at the basal lateral side, *Salmonella* are taken up by macrophages which is a crucial stage of infection. These macrophages have been activated via the secretion of inflammatory cytokines such as TNF-α, IFN-γ and IL-17 at the site of infection (Kurtz et al., 2017). Stimulation by this and bacterial antigens such as LPS, triggers iNOS to form NO (Aktan, 2004). This toxic radical is used to severely damage bacterial cells. Once inside the macrophage, *Salmonella* are able to modify the vacuole compartment, forming an SCV which entails expression of SPI-2 and effectors which alter cellular signalling (Waterman and Holden, 2003, Jennings et al., 2017). *Salmonella* encounter RNS and ROS within the hostile cell despite the protective SCV. Therefore, upregulation of detoxification genes are essential for their survival and allowing them to replicate.

Three metalloenzymes in particular have been thoroughly investigated in the literature for RNS detoxification in both *Salmonella* and more commonly, *E. coli*. These are HmpA, NrfA and NorVW which are highly regulated for the energetically expensive process of detoxifying NO (Prior et al., 2009). It is a well-known fact that *Salmonella* are more resistant to NO which leads to higher levels of intracellular survival than the closely related *E. coli*. Speculation for this surrounds additional detoxification mechanisms that are *Salmonella*-specific. In fact, the identification of STM1808 which was originally labelled for tellurite resistance on the *Salmonella* genome, was found to be upregulated by exposure to physiologically relevant

concentrations of NO (Karlinsey et al., 2012). Therefore, it is possible other proteins that have not been investigated as extensively which could also add to NO protection.

The fact that STM1808 was proven to also have a role in tellurite resistance, more investigation was directed to related protein YeaR which contains the same DUF1971 homology domain. Interestingly, *yeaR* was also found to be of importance in response to NO (Arkenberg, 2013) and tellurite resistance (Johnston, 2017). The other well-known tellurite resistance protein TehB may also be crucial for the nitrosative stress response. This thesis sought out to determine if STM1808, YeaR and TehB were important for tellurite resistance in the presence of low concentrations of K_2TeO_3 and to uncover if they was a functional overlap in these proteins for tellurite resistance and NO detoxification. Furthermore, to understand how conserved these proteins are among other serovars that are host specialised like *S*. Typhi, *S*. Dublin and *S. bongori* (detailed in Chapter 3).

6.2. STM1808, YeaR and TehB: not only responsible for tellurite resistance

As stated above STM1808, YeaR and TehB are all tellurite resistance proteins and interestingly they appear to be conserved among *Salmonella* investigated in this study (*S.* Typhi, *S.* Dublin and *S. bongori*). These were investigated as *S.* Typhi, is host restricted to causing disease in humans and it has genetically diverged from Typhimurium. For example, it does not contain the *spv* genes, an element required for *S.* Typhimurium to elicit systemic infection in mice and neither does *S. bongori*. But *S. bongori* branched off much earlier meaning it has a considerably basic set of virulence genes in comparison. Interestingly, *S.* Dublin does have these genes, but this pathogen is more well known for eliciting a unique respiratory and systemic infection in cattle. This thesis highlighted that all three proteins had homologs in all serovars investigated and they had high levels of identity to the versions identified Typhimurium. Amino acid substitutions were unlikely to have caused disruption to protein function. This proof substantiates that these tellurite resistance proteins are important and potentially for other metabolic functions. However, the relevance of *Salmonella* containing such tellurite resistance determinants is not entirely known and this is why these proteins were investigated for other aspects of *Salmonella* pathogenesis and survival.

In this thesis, these proteins were investigated specifically for NO detoxification. It was hypothesised that because they shared regulation by NsrR which controls other NO detoxification genes, meant it was likely these proteins were involved in NO detoxification. Previous studies were conducted to identify the core NO regulon of *Salmonella* which identified significant upregulation of *STM1808*, *yeaR* and *tehB* during exposure to NO among other genes which were controlled by NsrR and FNR (Arkenberg, 2013, Karlinsey et al., 2012).

They confirmed previous studies about the nitrosative stress pathway and what genes are upregulated which were conducted in *E. coli* (Justino et al., 2005, Mukhopadhyay et al., 2004).

This present study confirmed that all three proteins are required for tellurite protection in *Salmonella* Typhimurium. There was a functional redundancy observed for their roles in tellurite resistance, but it appeared as if TehB was most crucial for aerobic defence against tellurite toxicity whereas STM1808 was more important for anaerobic protection. Curiously, addition of tellurite to *ΔyeaR* did not have as much of an effect, which was similar to *ΔSTM1808*, further highlighting the functional overlap between the proteins. Astonishingly, when it came to NO, STM1808 was most crucial for aerobic protection against NO. However, anaerobically, all deletion mutants were attenuated signifying that all were equally important for anaerobic NO detoxification.

Of conditions tested here, more work could be done for anaerobic tellurite and NO exposure especially as during anaerobic conditions, the respiration of *Salmonella* changes drastically in order to use alternative electron acceptors. Through this truncated denitrification, NO is produced as a by-product, so detoxification is more crucial and enzymes responsible are put under larger strain.

6.2.1. Consequences of evolving tellurite resistance in *Salmonella*

Tellurite resistance mechanisms have been largely discussed already. There has been many genes identified in a range of organisms. For example, the *ter* operon (Lewis et al., 2018), *tehAB*, *kilA*, *telAB* (Taylor et al., 1994, Goncharoff et al., 1991, Turner et al., 1994), *txr* (Yasir et al., 2020) and *cysK* (Vasquez et al., 2001). A review on tellurite resistance conducted by Taylor (1999) showed that these genes were present in numerous species including *E. coli*, *Salmonella* Typhimurium, *Pseudomonas syringae*, *Rhodobacter sphaeroides*, *Yersinia pestis*, *Mycobacterium tuberculosis*, *Streptococcus pneumoniae* and many others. All of the genes that were identified at the time required strong upregulation or a large quantity of mutations to result in tellurite resistance and it was hypothesised that such genes possess alternative metabolic roles which have enabled their retention. This could therefore be true for *Salmonella* tellurite resistance genes. To further investigate the possibility of having ulterior functions this thesis pursued the investigation of generating high levels of tellurite resistance in a WT background and in a strain that lacked the copies of *Salmonella* tellurite resistance genes (*STM1808*, *yeaR*, *tehB*) (Chapter 4). It was hypothesised this could be done in WT as natural resistance could be built upon and a similar study was conducted in *E. coli* (Morales et al., 2017).

Developing tellurite resistance in the triple deletion strain was postulated to be more difficult to obtain. But evolving resistance was successful, and it supports the prediction of other enzymes being responsible for tellurite resistance or these genes having dual functionality. Such strains were then tested for alternative phenotypes, particularly focusing on NO, H_2O_2 and antibiotic resistance profiles. As if these profiles were altered, then overlap of these systems must be present (investigated in Chapter 4).

This was investigated because the mode of action of tellurite is still debated in the literature. Until recently, it was understood that reactive oxygen species such as H_2O_2, singlet O_2, $O_2^{\cdot-}$ and $OH^{\cdot}$ were the main causes of lethality during tellurite exposure (Pérez et al., 2007, Zannoni et al., 2007). This is generally still the case but more recently tellurite was linked to disrupting the haem biosynthetic pathway which creates accumulation of protoporphyrin IX as well as reducing cellular thiols and eliciting dangerous ROS (Morales et al., 2017). These can be detoxified by SODs (Wang et al., 2018) and other counteractive measures include antioxidant thiols such as glutathione which can relieve oxidative stress, but these are actively targeted by tellurite (Turner et al., 2001). However, it is not known whether tellurite directly causes this accumulation or if it tampers with enzymes. It is known to destroy Fe-S clusters (Morales et al., 2017), which similarly occurs during nitrosative stress (Tucker et al., 2008). This also causes a similar cascade whereby liberated Fe^{2+} can generate $OH^{\cdot}$ with H_2O_2 and these radicals are extremely damaging to DNA, proteins and the cell membrane (Rhen, 2019). Developing tellurite resistance could help to identify the targets in bacterial cells during tellurite exposure. This is because during directed evolution, there are typically changes in such targets that mediate the resistance phenotype. Another reason would be to discover any links between tellurite resistance and nitrosative or oxidative stress.

This study revealed that tellurite resistance could be developed in both strains, but toxicity of tellurite was replaced by toxicity for reduced tellurium which would accumulate in the cells as there are no ways for *Salmonella* to transport it out. The tellurite resistance strains did not have a significantly altered NO profile but interestingly their H_2O_2 profiles were changed. As a consequence of the mutations the WT evolved tellurite resistant strain was more resistant to up to 100 mM H_2O_2 and this was more prominent aerobically. The opposite was observed in the triple tellurite resistant strain which had increased sensitivity to H_2O_2. In the case of antibiotic resistance, both strains experienced increased sensitivity to gentamicin and ceftriaxone. However, with polymyxin B, the results were inconclusive and should be examined further. As NO tolerance in the tellurite resistant strains remained unchanged from parental strains, the survival within macrophages was assessed. Surprisingly, the reduction in Cfu/mL recovered did not differ significantly between WT and its tellurite resistant counterpart.

Suggesting that tellurite resistance gained in this strain may have been at the cost of particular virulence mechanisms.

Without knowing the genetic background of these tellurite resistant strains developed here, it is difficult to definitively state what has caused their phenotypes. Therefore, studying the differences on a genotypic level by searching for the presence of SNPs or other changes to the DNA should be considered in future. In order to further substantiate this, the directed evolution approach could be adapted to enable data collection on mutations after each passage, which would help refine suspected mutations responsible for tellurite resistance and the other phenotypes seen.

6.3. Solving the enigma within the nitrosative stress response: YgbA in *Salmonella*

The last investigation for this thesis surrounded the nitrosative stress response proteins YtfE, Hcp, YgbA and HmpA and their roles for tellurite protection. Additionally, the lack of phenotypic studies on *ΔygbA*, stimulated further investigation for the importance of YgbA in NO detoxification and for *Salmonella* survival within macrophages (detailed in Chapter 5).

It was hypothesised that the deletion of *ytfE* would impact anaerobic survival during tellurite exposure as this was previously reported (Johnston, 2017). There is no evidence in the literature that would suggest the deletion of *hcp* would greatly impact the mutant during tellurite exposure. Although as Hcp works with an NADH reductase, the deletion could have disrupted its redox partner during stress elicited by tellurite. Therefore, tellurite should negatively impact *Δhcp* growth. As for HmpA, it is not known to have a phenotype for tellurite presence but in the presence of NO has been recorded (Gilberthorpe et al., 2007). The same effect of NO on *ΔhmpA* was recorded in this study. The importance was proposed to extend to tellurite in assuming that mutants will display growth deficiency. This study revealed that HmpA was important for aerobic protection against tellurite. However, anaerobically, YtfE was most depended on in this study. Hcp did not appear to have a role in tellurite resistance. In order to corroborate these findings, the expression of these genes could be analysed during tellurite exposure.

It was hypothesised that deletion of *nsrR* would improve the strains ability to respond to NO and tellurite. This was believed to happen as the repressor would no longer be there to control expression of NO detoxification and tellurite resistance genes. In previous studies when this was done, researchers noticed that the strain would not perform well in macrophages but had little difference in growth *in vitro* (Gilberthorpe et al., 2007, Karlinsey et al., 2012). This was due to *hmpA* needing strict expression patterns during macrophage infection to handle the

oxidative and nitrosative bursts. It has been shown here that the deletion of *nsrR* causes attenuation during aerobic tellurite exposure, but this is less than seen in WT and no difference was seen anaerobically. This phenotype was reversed during exposure to deta NONOate. This phenomenon is caused by the de-repression of tellurite resistance genes and NO detoxification genes, but genes were also under control by other regulators. In order to investigate which genes remained repressed, expression studies could be performed in future.

The deletion of *ygbA* was hypothesised to be detrimental for cells undergoing nitrosative stress. This would include exposure to NO donors or during macrophage infection, because *ygbA* was highly upregulated during exposure to NO donors in other studies in *E. coli* (Mukhopadhyay et al., 2004, Justino et al., 2005) and *Salmonella* (Gilberthorpe et al., 2007, Karlinsey et al., 2012). With other authors predicting this hypothetical protein to have a role in aerobic protection against RNS (Arkenberg, 2013). However, there is no data on how this mutant would perform during tellurite exposure. If this NO detoxification gene was like others, it may also have another function for tellurite resistance as overlap was found in Chapter 3 for all three tellurite resistance proteins. Following this it could be predicted that there would be a negative effect of tellurite on *ΔygbA*. During tellurite exposure, there were little differences in how the strain grew aerobically compared to WT. However, anaerobically YgbA appeared to provide a small amount of protection against tellurite. In this present study, YgbA was shown to not be important for anaerobic or aerobic exposure to 5 mM deta NONOate. Whereas in macrophages, the importance for YgbA was evident as significantly lower Cfu/mL of *ΔygbA* were recovered from macrophage lysates compared to WT. A similar result was recorded by Arkenberg (2013), but interestingly the differences were minimised in IFN-γ macrophages. This experiment could be repeated again with using other cytokines that are known to activate macrophages such as TNF-α or a combination of both treatments which would mimic the host immune response.

6.4. Clinical context

Salmonella Typhimurium TTSS encoded by SPIs do not target mature macrophages (Geddes et al., 2007) and preferably replicate within neutrophils which get recruited to a localised site of inflammation in the gut mucosa (Garai et al., 2012). Neutrophils have higher concentrations of ROS which are used to elicit antimicrobial or inhibition of growth (Winterbourn et al., 2016). However, the reliance on macrophages are typically employed for the clearance of systemic Typhoid fever (Forest et al., 2010) and have much higher concentrations of RNS such as NO due to iNOS (Aktan, 2004, Henard and Vazquez-Torres, 2011). As *S.* Typhimurium elicit a typhoidal infection in mice, infecting RAW 264.7 murine macrophages are a useful model for the study of *S.* Typhi. Therefore, the use of this cell line for gentamicin protection assays meant

results gained applied more to systemic typhoid infection seen in mice. However, *in vitro* experiments that mimic neutrophils with high concentrations of H_2O_2 and low concentrations of NO would apply more physiologically to gastroenteritis. Although it should be noted that conducting *in vitro* assays do not directly apply *in vivo* because of multiple variables that cannot be exact to true conditions.

Despite the fact there is limited evidence on *Salmonella* encountering tellurite, the point that can be made is that resistance genes for tellurite are present in this zoonotic pathogen. Furthermore, antibiotic resistance genes with heavy metal resistance is becoming more prominent (Mourao et al., 2015, Figueiredo et al., 2019). So, although it is unlikely that evolved tellurite resistant strains would be the cause of clinical infections, it is essential to investigate how co-resistance can impact the virulence of microbes. This is because the mechanisms used for tellurite resistance have additional purposes, such as resistance to ROS and RNS, that enable successful infection in hosts. Therefore, the investigation of unlikely characteristics may give researchers additional avenues for the exploration of new antimicrobials for the treatment of MDR *Salmonella* or alternative mutations that could be used in vaccination strains.

This study has investigated the link between tellurite resistance and nitric oxide. This has also included the role of ROS such as H_2O_2 because of how tellurite toxicity is thought to mediate in bacterial cells. Based on the findings here in the evolved tellurite resistant strains the link is more prominent between ROS and tellurite resistance. However, further evidence is required to disseminate phenotypic and genetic links between RNS and tellurite resistance because mutations acquired in these evolved strains could identify other genes that share physiological functions. The results from Chapters 3 and 5 however, still justify an *in vitro* correlation between NO detoxification and tellurite resistance proteins in the presence of low concentrations of NO and tellurite.

6.5. Study Limitations

Further investigation should be conducted in minimal media to assess any differences between strains for both tellurite and deta NONOate, as the present study used LB which a high nutrient growth media. It would be interesting to see if the same patterns emerged in nutrient deprived minimal media. Furthermore, currently there is a lack of investigation in complementation strains which contain a WT copy of the gene on a plasmid. If this could be done, it would validate that phenotypes detected were caused by the deletion of that particular gene. Another limitation to this investigation is the lack of genotypic data from directed evolution studies as well as other phenotypes being assessed. Again, this would have been easier to use bioinformatics analysis to lead phenotypic characterisation. Therefore, this

should be conducted in future. Moreover, additional serovars should be investigated further for the conservation studies of STM1808, YeaR and TehB as it could give useful information on when serovars diverged from one another.

6.6. Final thoughts and conclusions

This thesis has emphasised the importance of the nitrosative stress response within *Salmonella* for exposure to NO and the overlap of tellurite resistance. It was brought forward that conservation of tellurite resistance genes and roles identified in NO protection justify their propagation in the *Salmonella* genome. Moreover, the fact that well known NO detoxification genes such as HmpA and YtfE were found to give protection against tellurite proves the correlation between the two responses. Physiologically, these proteins that are additionally capable for tellurite resistance (although mechanisms undetermined) also enable the survival of *Salmonella* against nitrosative and to an extent oxidative stresses. Therefore, enabling the pathogenesis of this enteric pathogen to uniquely invade and colonise macrophages. The result that YgbA contributes to survival within macrophages and importance of this protein anaerobically during exposure to an NO donor proves its value in nitrosative stress but can also indicate this protein having roles in protection for a number of stresses elicited in macrophages. Furthermore, evolving tellurite resistant *Salmonella* has stimulated the discussion tellurite resistance mechanisms, their uses in this enteric pathogen, how this bacterium mediates transport of tellurite or tellurium, and which mutations are responsible for causing such phenotypes.

In conclusion, this study builds on our current knowledge of *Salmonella* NO detoxification and tellurite resistance strategies and how these systems have several genes that overlap which enable functional redundancy. Further work is required to validate these findings and potential avenues have been described here. Understanding these processes build on knowledge of *Salmonella* pathogenesis which could ultimately enable researchers to discover potential drug targets to combat the global problem of *Salmonella* infections.

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