Salmonella Typhimurium: Mechanisms of nitric oxide detoxification and regulation of the periplasmic nitrate reductase

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Abstract

The enteric pathogen *Salmonella* causes both severe systemic infection and acute gastroenteritis, commonly known as food poisoning, in humans. It is therefore responsible for millions of food and water born infections worldwide each year and has a significant burden on global health. Typhoid fever is a serious, life-threatening illness while gastroenteritis caused by *Salmonella* is often fatal to at-risk individuals such as the immunocompromised, the elderly and infants. The human innate immune system combats *Salmonella* infection with both reactive oxygen and reactive nitrogen species (ROS and RNS) largely produced by macrophages. Both ROS and RNS are capable of damaging bacterial DNA, lipids and proteins, inhibiting *Salmonella* replication and survival. Nitric Oxide (NO), a free radical and cytotoxin, is one such RNS produced by the host immune system that acts as an antibacterial defence against *Salmonella*. In order to survive in the host *Salmonella* must therefore employ mechanisms to detoxify NO it encounters both endogenously and exogenously. Currently three enzymes are well-characterised as mechanisms by which *Salmonella* detoxifies NO, however *Salmonella's* ability to survive without these three enzymes suggests the existence of other, yet unidentified, NO detoxification genes.

In this study, various *Salmonella* genes shown to be up-regulated under nitrosative stress in preliminary data were further investigated and their role in NO detoxification characterised. Single and combination deletion strains were constructed and resultantly showed sensitivity to both nitrosative and oxidative stress. This study also examined the regulation of the periplasmic nitrate reductase of *Salmonella*, NapA, in relation to the SPI-1 transcriptional activator HilA. Results of this research implicate a role for HilA as a negative regulator of *napA* expression.

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Abbreviations

[Fe-S]	Iron Sulphur Cluster
Amp	Ampicillin
AMPs	Antimicrobial proteins
ATR	Acid Tolerance Response
Bp	Base Pair
Cat	Chloramphenicol acetyltransferase
Cm	Chloramphenicol
CBC	Crypt base columnar
CDC	Centre for Disease Control and Prevention
$CHCl_3$	Chloroform
cDNA	Complementary DNA
Cfu	Colony Forming Units
°C	Defrees centigrade
DC	Dendritic Cells
dH_2O	Distilled water
DUF	Domain of unknown function
DNA	Deoxyribunucleic Acid
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EFIs	Extraintestinal focal infections
eNOS	Endothelial Nitric Oxide Synthase
FLP	Flippase Recombinase
FNR	Fumurate and nitrate reductase regulator
Fur	Ferric uptake regulator
F/S	Filter sterilized
G	Grams
GALT	Gut associated lymphoid tissue
GEF	Guanine exchange factor
GFN	Global Food-borne Infections Network
GI-Tract	Gastrointestinal tract
GOI	Gene of interest
GTP	Guanosine-5'-triphosphate
H	Hydrogen
H_2O_2	Hydrogen Peroxide
HNO_2	Nitrous Acid
HilA	Hyperinvasion Locus Protein A (transcriptional activator)
H-NS	DNA Binding Nucleoid Associated Protein
HmpA	Flabohemoglobin
IFN-γ	Gamma interferon
IgG	Immunoglobin G
IL	Interleukine

iNOS	Inducible Nitric Oxide Synthase
iNTS	Invasive non-typhoidal *Salmonella*
IRF	Interferon Regulator Factor
JAK	Janus Kinase
Kb	Kilobase-pair
Km	Kanamycin
LB	Luria-Bertoni
LBP	LPS-binding protein
LPS	Lipopolysaccharide
µg	Micrograms
µl	Microlitres
µM	Micromolar
M	Molar
M cells	Microfold cells
MDR	Multi-drug resistant
ml	Milliliters
mM	Millimolar
MOI	Multiplicity of infection
N^+/G^-	Nitrate-sufficient/glycerol-limited
N^-/G^+	Nitrate-limited/glycerol-sufficient
N_2	Dinitrogen
N_2O	Nitrous oxide
N_2O_3	Dinitrogen trioxide
NAD+	Nicotinic Adenine Dinucleotide
NADH	Nicotinic Adenine Dinucleotide (reduced)
NAD(P)+	Nicotinic Adenine Dinucleotide Phosphate
NAD(P)H	Nicotinic Adenine Dinucleotide Phosphate (reduced)
NFκB	Nuclear factor-kappaB
NH_4^+	Ammonium
NO	Nitric Oxide
$NO_2^{\cdot}$	Nitrogen dioxide radical
NO_2	Nitrate
NO_3	Nitrite
NorR	Nitric oxide reductase transcriptional regulator
NorV	Flaborubedoxin
NrfA	Cytochrome *c* nitrite reductase
NsrR	Nitric oxide-sensitive repressor
NTS	Non-typhoidal *Salmonella*
O_2	Oxygen
$O_2^{\cdot -}$	Superoxide anion
OD	Optical density
$OH^{\cdot}$	Hydroxyl radical
ONPG	*o*-nitrophenyl-β-D-galactopyranoside

$ONOO^-$	Peroxynitrate
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PMN	Polymorphonuclear leukocytes
Ppm	Parts-per-million
PP	Peyer's Patches
RNA	Ribonucleic acid
RNI	Reactive Nitrogen Intermediates
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
Rpm	Revolutions Per Minute
***S.* Typhimurium**	*Salmonella enterica* serovar Typhimurium
SCV	*Salmonella* Containing Vacuole
SPI-1	*Salmonella* Pathogenicity Island 1
SPI-2	*Salmonella* Pathogenicity Island 2
STAT	Signal Transducer and Activator of Transcription
TA	Trans-amplifying
TLR	Toll-like Receptor
TNF-α	Tumor necrosis factor alpha
TTSS	Type Three Secretion System
Vi	Virulence capsular polysaccharide of *S.* Typhi
V	Volts

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For Andy, who is dearly missed.

CHAPTER 1: INTRODUCTION

1.1 *Salmonella*

1.1.1 Physiology

Salmonella are gram-negative, rod-shaped bacteria of the *Enterobacteriacae* family (Prior *et al*, 2009). These microorganisms range from 0.7 to 1.5μm in diameter and 2-5μm in length with peritrichous flagella which allow for their high motility (Fàbrega & Vila, 2013). As with other Gram-negative bacteria, *Salmonella* possess a thin peptidoglycan layer between their inner cytoplasmic and outer cell membrane. A major component of this outer cell membrane is lipopolysaccharide (LPS), which is capable of activating a variety of host-immune responses. A constituent of LPS is the O-polysaccharide or antigen which is used in conjunction with the bacterial flagellin proteins (H1 and H2 antigens) to differentiate between serotypes (McQuiston *et al*, 2011). As a facultative anaerobe *Salmonella* is capable of survival and growth in either aerobic or anaerobic conditions, a characteristic which contributes to its success as an enteric pathogen (Rowley *et al*, 2012).

1.1.2 Nomenclature

There are over 2,500 serovars of *Salmonella* currently characterized, with new serovars identified each year (Popoff *et al*, 2004). All recognized serovars belong to one of two species, *Salmonella bongori* or *Salmonella enterica* (Fàbrega & Vila, 2013) (Figure 1). *Salmonella* nomenclature here becomes considerably more complex, as both species may be divided further into subspecies on the basis of the Kauffmann-White scheme (Fàbrega & Vila, 2013; Su & Chiu, 2007). The subspecies *S. bongori* consists of only 22 serovars that rarely infect humans and are predominately found in reptiles or

in the environment (Fàbrega & Vila, 2013). In comparison, *S. enterica* consists of six subspecies, including *S. enterica* subspecies enterica (I) that contains almost all serovars which cause disease in domestic animals and humans (Grimont & Weill, 2007; Fàbrega & Vila, 2013). Strains belonging to the species *S. enterica* may also be classified into 67 serogroups on the basis of their O surface antigen or, when differentiated by both O and H antigens, into 2,557 serovars. Of these, 1,531 serovars are found in the *S. enterica* subspecies *enterica* and are usually named for the geographical region in which they were identified (Fàbrega & Vila, 2013, Popoff *et al*, 2004). Exceptions include the serovars *Salmonella enterica* ssp. *enterica* serovar Typhi (*S*. Typhi), *Salmonella enterica* ssp. *enterica* serovar Paratyphi (*S*. Paratyphi A, B and C), *Salmonella enterica* ssp. *enterica* serovar Typhimurium (*S*. Typhimurium) and *Salmonella enterica* ssp. *enterica* serovar Enteritidis (*S*. Enteritidis) all of which are prominent causes of infections in humans (Fàbrega & Vila*,* 2013). Serovars display varied host adaption and restriction, such as *S.* Typhi, *S.* Paratyphi A, B and C, which exclusively infect humans and some nonhuman primates to cause enteric fever (Pascopella *et al*, 1995). However, most strains have a broad host range and are able to infect both animals and humans, though the disease caused may vary according to the host. For instance, *S.* Typhimurium is a cause of acute gastroenteritis in humans but, as its name suggests, causes a systemic disease similar to Typhoid in mice (Suar *et al*, 2006). *S.* Dublin is adapted to cattle, where it is capable of causing systemic and enteric disease, whilst in humans *S.* Dublin is an infrequent cause of septicemia. Furthermore, in laboratory settings *S.* Dublin was shown to cause Typhoid fever-like infections

in mice (Suar *et al*, 2006; Barrow *et al*, 1994). The ability of *Salmonella enterica* serovars to manifest as distinctive diseases in different hosts is a highly researched topic in which much remains to be elucidated.

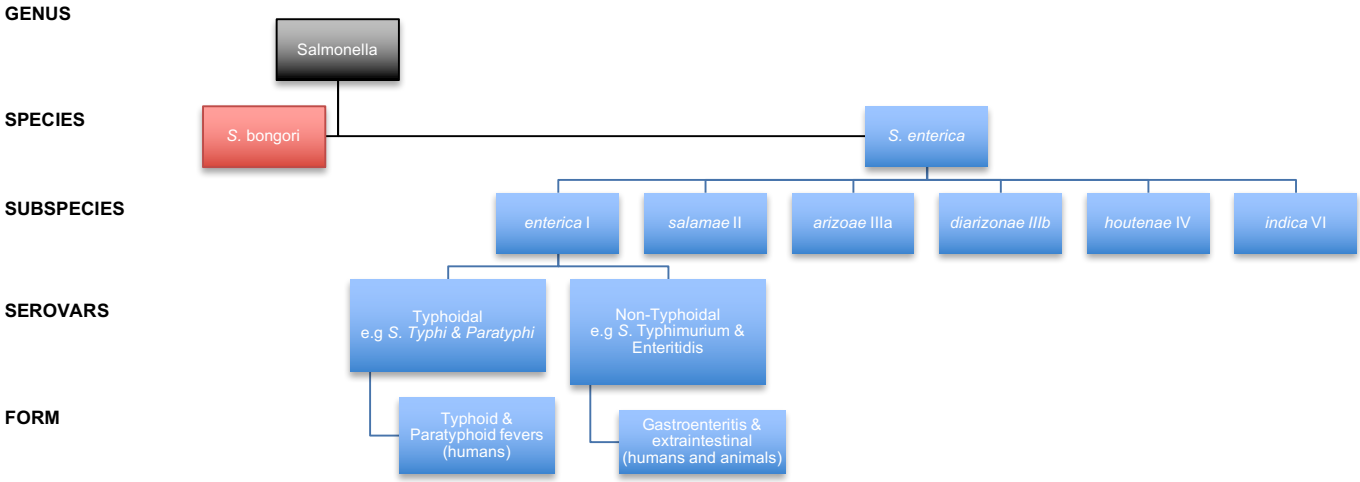


Figure 1: The *Salmonella* genus. The genus *Salmonella* may be divided into two subspecies, *S.* bongori and *S.* enterica. *S.* bongori contains five subspecies, whilst *S.* enterica contains six subspecies, which can be further divided into serovars on the basis of their O and H antigens. *S.* enterica contains all serovars which cause disease in domestic animals and humans. These serovars may be either Typhoidal, meaning that they cause Typhoid fever in humans, or non-Typhoidal serovars, which only cause gastrointestinal and extraintestinal infections in humans. (Figure adapted from Hurley et al, 2014.)

1.1.3 Epidemiology

In terms of clinical manifestation *Salmonella* serotypes cause one of three distinctive illnesses; Typhoid Fever, bacteremia or enteritis (Santos *et al*, 2001). Serotypes which cause Typhoid are therefore known accordingly as Typhoidal strains, whilst those that result in gastroenteritis and bacteremia are referred to as non-Typhoidal. Due to differences in disease recording between countries, and allowing for unreported and undiagnosed cases of *Salmonellosis,* statistics reflecting disease incidence are hard to come by and somewhat unreliable. However, it is thought that worldwide *Salmonella* kills some 1 million people each year (Crump *et al*, 2004). The current estimation stands at some 20-30 million cases and 200,000 deaths worldwide associated with Typhoid Fever annually (Wong *et al*; 2015, Zhang *et al,* 2008, Crump *et al*, 2004). Whilst the incidence of gastrointestinal infections associated with non-Typhoidal *Salmonella* (NTS) is estimated to be some 93 million cases each year (Majowicz *et al*, 2010). However, such figures may be highly conservative estimates due to the self-limiting nature of the majority of *Salmonella* strains; most infections in healthy persons are not reported or treated, as symptoms are non-life-threatening, with the individual recovering after 3-7 days. Demographics most at risk for infection include the elderly, children and the immunosuppressed, and *Salmonella* may cause severe complications or even fatality in these individuals. Whilst gastroenteritis is common worldwide, Typhoid fever is rare across Europe and the USA. It is however widespread throughout sub-Saharan Africa and Asia, with incidence being particularly high where sanitation is poor (Prior *et al,* 2009) (Figure 2).

Typhoid is therefore a prominent health concern for endemic people and for those travelling to such destinations.

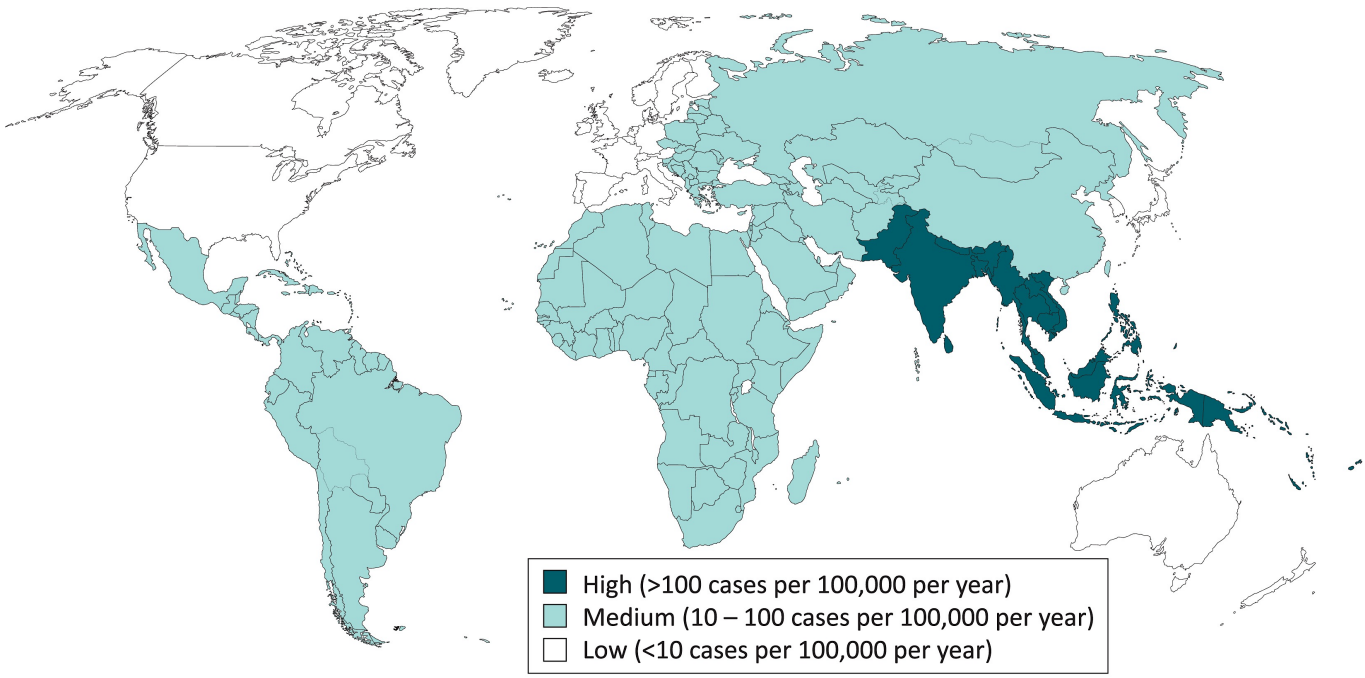


Figure 2: Epidemiology of Typhoid Fever. Map showing the distribution of *Salmonella* enterica Serotype Typhi (1990-2002) (Figure taken from Crump *et al*, 2004).

1.1.3.1 *Salmonella* Typhimurium

This study involves the use of *S.* Typhimurium, a serovar belonging to the *S. enterica* subspecies *enterica,* which is a significant cause of non-Typhoidal, acute gastrointestinal infection in humans. This particular serovar, as its name implies, is also capable of causing a systemic disease in mice similar to that of Typhoid and is therefore prominently used in the laboratory-based study of Typhoid and host-pathogen interactions (Santos *et al*, 2001; Suar *et al*, 2006). *S.* Typhimurium has a broad host range including not only rodents and

humans, but cattle, poultry and other mammals (Garai *et al*, 2012). Like the rest of its genus, *S.* Typhimurium has a wide temperature range with an optimum of 37°C, conferring the ability to survive and colonise a diverse assortment of environments. Also, as will be discussed in this study, *S.* Typhimurium has the ability to adapt and survive a multitude of stress factors, including, but not limited to, heat, oxidative and nitrosative stress.

1.1.4 Typhoid Fever

Typhoid Fever is a severe and systemic illness caused by host-adapted Typhoidal strains *S.* Typhi and *S*. Paratyphi and which has a mortality of 10-30% without treatment. Infection occurs most commonly via the fecal-oral route (Anwar *et al*, 2014). The bacteria enter through the intestine and are disseminated in the blood to the intestinal lymph nodes, liver and spleen where they multiply (Bäumler *et al*, 2008). Infected individuals typically present with chills, headache, sore throat, constipation or diarrhoea and myalgia prior to the onset of fever (Anwar *et al*, 2014). The illness progresses with increasing malaise, bacteraemia and diffuse enterocolitis. Enterocolitis in Typhoid is associated with hypertrophy which may lead to necrosis in Peyer's Patches (PP) and mesenteric lymphoid tissue. In severe cases necrosis and ischemia of the tissue results in perforation of the ulcerated Peyer's Patches and subsequent haemorrhage (Sirinavin & Garner, 2000; Everest *et al*, 2001). This may in turn lead to peritonitis, septicaemia and death (Prior et al, 2009; Everest *et al*, 2001). Gastrointestinal bleeding may also occur, and is seen in approximately 10% of patients (Parry *et al*, 2002). Individuals may display bradycardia, hepatomegaly, splenomegaly and 'rose spots'; bacterial emboli

which are typical of enteric fever and appear as a rash from as early as a week after infection. In 5-10% of cases the infection may manifest neuropsychiatrically, known as typhoid encephalopathy, with psychosis and confusion (Anwar *et al*, 2014). Diagnosis involves the detection of bacteria in the blood through PCR or culture (Hosoglu & Wain, 2008). The Widal test has been traditionally used as a method of detection for Typhoid and in some developing countries is the only laboratory means of diagnosis for enteric fever (Olopoenia & King *et al*, 2000). This serological test employs agglutination to demonstrate the presence of H and O *Salmonella* antigens in the serum of the infected individual. However, doubts have been raised about the accuracy of this test, which often produces false-positive results due to cross-reactivity with other infectious organisms. Positive results may also be caused by previously contracted enteric fever, typhoid vaccination or a response to antibodies present in populations where Typhoid is endemic. Furthermore, whilst the test is strongly positive in the later stages of infection, serological tests are inaccurate until approximately a week after initial infection (Younis *et al*, 2009). Newer diagnostic approaches have looked at the detection of circulating IgA in order to improve sensitivity and specificity (Sheikh *et al*, 2009). In 2004 it was estimated that the annual frequency of Typhoid was approximately 27 million worldwide (Crump *et al*, 2004; Anwar *et al*, 2014). Globally it is estimated that the annual number of deaths from Typhoid stands within the range of 200,000-800,000; based on the 1-4% mortality rate of invasive S. Typhi (Crump *et al*, 2004, Breiman *et al*, 2012, Steele *et al*, 2008). As such Typhoid is a significant health issue worldwide.

1.1.5 Gastroenteritis

Acute gastroenteritis in humans is caused by several *Salmonella* serovars, though *S.* Enteriditis and *S.* Typhimurium are most commonly isolated in clinical practice (Prior *et al*, 2009; Fábraga & Vila, 2013). In fact, the Global Food-borne Infections Network (GFN) suggests 80% of all human cases of NTS worldwide are attributable to *S.* Typhimurium or *S*. Enteritidis (Haeusler & Curtis, 2013).

The infectious dose for non-typhoidal strains of *Salmonella* ranges from 10^6-10^8 organisms for healthy adults, though infants, the elderly and individuals with underlying conditions are more susceptible (Chen *et al*, 2013). Globally, the estimated number of annual gastroenteritis cases resulting from NTS infection is 93.8 million, with some 155,000 deaths (Gal-Mor *et al*, 2014; Majowicz *et al*, 2010.) Infection occurs through the ingestion of contaminated food or water and is followed by an incubation period varying from 4 to 72 hours dependent on the host (Chen *et al*, 2013, Velge *et al*, 2005). The spectrum of severity for gastroenteritis is broad and may range from mild to acute forms. The infection presents with fever and chills, nausea and vomiting, abdominal cramping and diarrhoea (Chen *et al*, 2013). Diarrhoea may contain blood, mucus and lymphocytes but is usually self-limiting after a period of 3-7 days (Prior *et al*, 2009; Chen *et al*, 2013). As such infection rarely requires treatment, though in young, elderly or immunocompromised patients complications such as bacteraemia or extraintestinal focal infections (EFIs) may arise which require antimicrobial therapy (Chen *et al*, 2013; Prior *et al*, 2009; Chen *et al*, 2007). Bacteraemia occurs in 10-15% of infected individuals, and this may progress to focal infection such as meningitis, and

bone and joint infection (Chen *et al*, 2013). Acute gastrointestinal infections which lead to complications may sometimes prove fatal, particular in high-risk individuals. However, in most immunocompetent individuals recovery is prompt, though bowel habits may be effected for up to several months after the illness. Following infection *Salmonella* bacteria are shed in the faeces, usually for a period of 5 weeks, though this is sometimes prolonged in young children (Chen *et al*, 2013). This short-term asymptomatic excretion following infection is known as convalescent carriage, whereas periods of excretion lasting for more than a year is defined as chronic carriage (Haeusler & Curtis, 2013; Buchwald & Blaser, 1984).

1.1.6 Sources of Infection

Salmonella infection occurs most commonly through contaminated foods or water, although human to human and animal to human transmission is also possible (Prior *et al*, 2009). This latter form is known as zoonotic transmission, and in the case of *Salmonella* can occur from contact with cats, dogs, rodents and, in particular, reptiles or amphibians (Hohmann, 2001; Mermin *et al*, 2004; Braden, 2006; Haeusler and Curtis, 2013). Poultry and egg products are common sources of *Salmonella*, as well as beef and pork. (Muhlig *et al* 2014; Adak *et al*, 2005). However, fruits and vegetables may also act as reservoirs for *Salmonella.* For instance, recent *Salmonella* outbreaks have been associated with contaminated products such as sprouts, tomatoes, peanuts and spinach (Gal-Mor *et al*, 2014). In 2008 there was a severe outbreak of *Salmonellosis* in the US from jalapeño peppers (Mody *et al*, 2011). More recently, in 2014, there was an outbreak of *S.* Newport originating from

cucumbers which caused some 275 diagnosed illnesses in the US (Bayer *et al*, 2014). This highlights the risk that *Salmonella* poses for industrialized countries as well as low-income nations.

1.2 Disease Progression and Host-Pathogen Interactions

1.2.1 Physiology of the Host Gastrointestinal Tract

The mammalian gastrointestinal system functions primarily as a gateway by which nutrients and water can enter the body (Saffrey, 2014). As such it is exposed to a variety of enteric pathogens through the ingestion of food and water and is thus equipped with various methods of destroying these microorganisms. The first of these defences are the salivary lysozymes in the mouth that have the ability to cause bacterial cell lysis and function as part of the innate immune system. Enteric pathogens that survive are then exposed to the acidic environment of the stomach, which at a pH of 2 serves as an effective host-defence against microorganisms. In the immediate post-prandial period acidity has even been known to fall as low as pH1 (Levy *et al*, 2006; Rychlik & Barrow, 2005). Individuals with alchlorhydria, or those undergoing treatment with proton-pump inhibitors or large quantities of antacids are therefore more susceptible to infection due to a less acidic gastric barrier (Tennant *et al,* 2008). A further defence is nitric oxide (NO) produced in the stomach from dietary and salivary nitrate. At pH2 and lower reactive nitrogen species (RNS) such as NO are directly bactericidal (Bourret *et al*, 2008; Henard & Vázquez-Torres, 2011). Any bacteria that survive the considerable obstacle of the stomachs acidic environment and RNS then move into the small intestine by the pyloric sphincter. Here, bile salts and

pancreatic juices released in the gut lumen cause a rise to an alkaline pH, preventing acidic damage to the intestine and aiding in the absorption of various nutrients and vitamins (Levy *et al* 2006). The small intestine itself is some 16 feet long with a 1-inch diameter and an exceptionally large surface area owed to the presence of villi and microvilli. (Gracz & Magness, 2014). The small intestine may also be differentiated into sections, with the beginning known as the duodenum, the middle is referred to as the jejunum and the final part of the small intestine, the ileum (Santaolalla *et al*, 2011). It is a multi-layered tissue, composed of the intestinal mucosa, submucosa, muscularis and serosa. The intestinal mucosa is composed of the intestinal epithelium and the lamina propria. The intestinal epithelium itself lines the surface of the small intestine and acts as a vital barrier between the intestinal lumen and the rest of the body. It is a crypt-villus structure composed of six differentiated epithelial cell types all originating from a common stem cell progenitor. The stem cells of the small intestine are the crypt base columnar (CBC) cells, which are interspersed between Paneth cells at the bottom of the crypt. These stem cells underdo asymmetric division, with one daughter cell replacing the original stem cell at the crypt base; whilst the other goes on to become a progenitor cell committed to producing a particular mature cell lineage. This progenitor cell moves up out of the crypt and trans-amplifying (TA) compartment and matures into a specialized epithelial cell type as it migrates to the top of the villus. Once at the top of the villus the mature cell undergoes apoptosis and is shed into the gut lumen. This process of continuous proliferation of epithelial cells from the crypt base and migration to the villus apex allows for rapid and constant renewal of the epithelium of the small

intestine. On average the intestinal epithelium is renewed every 4-5 days (Clevers, 2011). The cell types comprising the intestinal epithelium include enterocytes, which posses a luminal brush border, goblet and enteroendocrine cells which secrete mucus and hormones respectively, tuft cells which sense luminal contents and Paneth cells which reside near the bottom of the crypt and provide the stem cell niche. Paneth cells are important for their role of secreting lysozymes, defensins and other bactericidal products that serve as a defence against enteropathogenic colonisation. Finally, microfold (M) cells are uniquely localized to the specialized-epithelium covering the lymphoid follicles within the wall of the small intestine known as Peyer's patches (PP). PP are aggregated lymphoid nodules which have a critical role in mucosal immunity; together with the appendix, lymphoid follicles and mesenteric lymph nodes, they form the gut associated lymphoid tissue (GALT). PP are dome-shaped and situated in the small intestinal wall, being most numerous near the terminal ileum (Cornes, 1965). These follicles lack goblet cells and therefore have a reduced mucus layer, instead they are largely covered by columnar epithelial cells and contain the aforementioned M cells. M cells lack microvilli on their apical surface, instead having broader microfolds. They also contain a deep, intraepithelial cavity which houses a variety of mononuclear cells (Kucharzik *et al*, 2006). M cells are proposed to act as an antigen sampling system by serving as a gateway through which luminal antigens are transported to the intestinal immune system, allowing an immune response to be mounted if necessary (Clevers, 2011; Zoetendal *et al*, 2006). Whilst M cells are important for inducing an effective immune response in the gut, they also present a weak point in the intestinal epithelial barrier

through which invasive pathogens, such as *Salmonella*, may invade the body (Kucharzik *et al*, 2006).

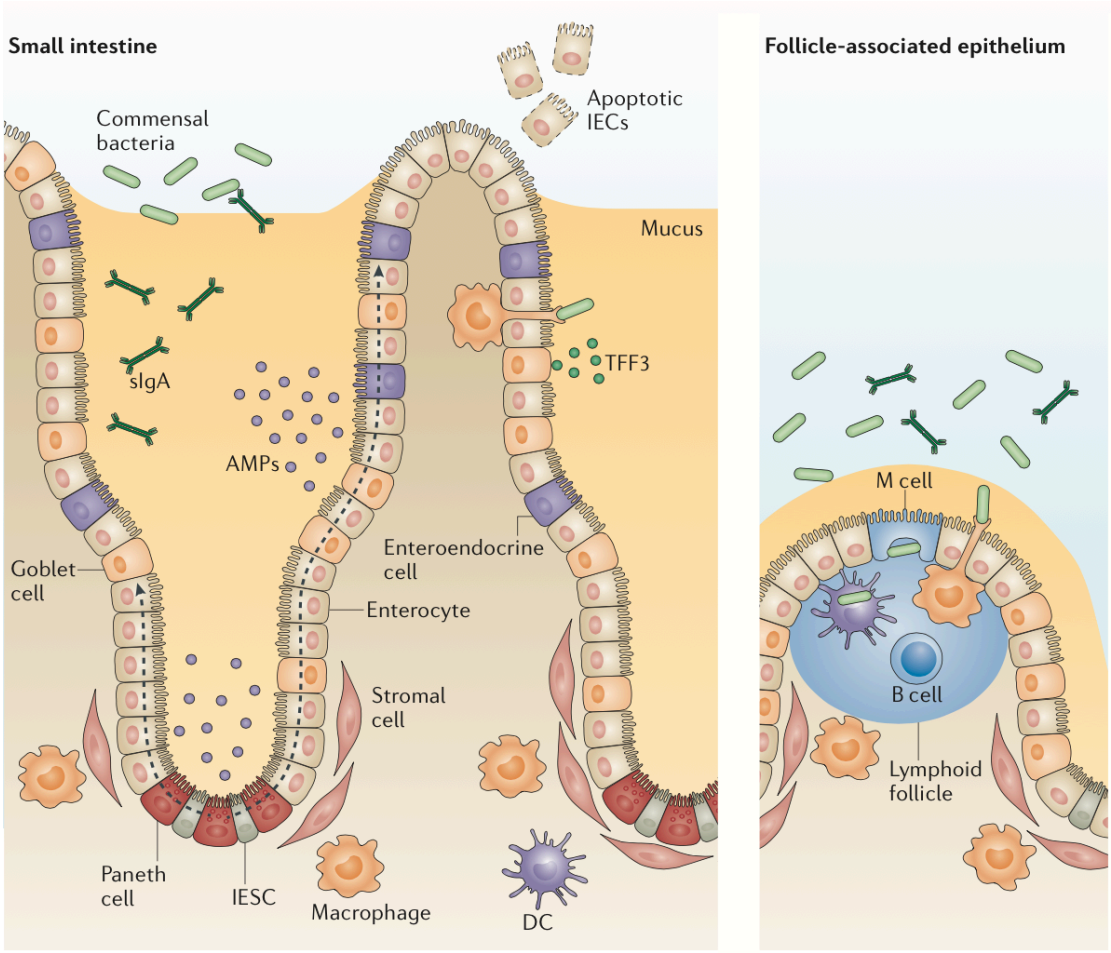


Figure 3: Morphology and structure of the intestinal epithelium. The intestinal epithelium is a crypt-villus structure. Intestinal epithelium cells (IEC) create a barrier between luminal microbiota and the mucosal immune system. Intestinal epithelial stem cells (IESC) reside at the base of the crypt in the niche maintained by Paneth cells and undergo asymmetric division to allow continuous renewal of the epithelial cell layer. With the exception of Paneth Cells which move to the bottom of the crypt, progenitor cells migrate up the crypt-villus axis and become differentiated IECs, such as Globlet cells, enterocytes or enteroendocrine cells. Once these cells reach the villus axis they undergo apoptosis and are shed into the lumen. Goblet cells and Paneth cells secrete mucus and antimicrobial proteins (AMPs) in an effort to exclude and combat pathogens on the epithelial surface. Secretory IgA (sIgA) is also released into the lumen as a defence against pathogens. Microfold cells (M cells) mediate the movement of antigens in the gut lumen across the epithelial barrier to Peyer's Patches and associated dendritic cells (DC) and macrophages. (Figure taken from Peterson & Artis, 2014).

1.2.2 Infection Process

The infectious dose of *Salmonella* varies depending on bacterial strain, the physiological health of the host and the food with which the bacteria are consumed (Prior *et al*, 2009; Darwin and Miller, 1999). The first stage of *Salmonella* infection involves survival of the early gastrointestinal tract to successfully reach the host intestine where it can then begin the invasive stage of infection during which it infiltrates the host intestinal epithelium.

1.2.2.1 Gastrointestinal Phase

Once ingested, *Salmonella* is exposed to the acidic environment of the stomach which at a pH of 3-4 serves an effective host defence against *Salmonella* survival (Prior *et al*, 2009) In the immediate post-prandial period acidity has even been known to fall as low as pH1 (Levy *et al*, 2006; Rychlik & Barrow, 2005). A further defence is nitric oxide (NO) produced in the stomach from dietary and salivary nitrate. At pH2 and lower reactive nitrogen species (RNS) such as NO are directly bactericidal against many micro-organisms (Bourret *et al*, 2008; Henard & Vázquez-Torres, 2011). In order to survive this acidic environment *Salmonella* switches on genes initiating an Acid-tolerance Response (ATR). The ATR is triggered in *Salmonella* by exposure to acidic foods or environment and enhances the organism's resistance to acidity. The expression of more than 50 acid shock proteins are involved in the ATR, controlled by a variety of signalling pathways (Bourret *et al*, 2008). Any bacteria that survive the considerable obstacle of the stomach's acidic environment and RNS then move into the small intestine by the pyloric sphincter. They must then survive killing by digestive enzymes, bile salts,

RNS, antimicrobial peptides, secretory IgA and other innate immune defences in order to reach the underlying epithelium (Haraga *et al,* 2008). Here *Salmonella* encounters further host-resistance in the form of the gut commensal flora, which act competitively against enteropathogenic colonisation (Fàbrega & Vila, 2013). Current data seems to suggest that *Salmonella* may outcompete this high density of commensal microbiota as a result of gut inflammation which acts beneficially for the pathogen. In brief, inflammation caused by the host-response appears to confer a growth advantage upon *Salmonella*, as nutrient access becomes limited. The pathogen, being motile and capable of chemotactic movement is able to obtain the high-energy nutrients localized by mucosal inflammation, leading to out-replication and growth over gut microbiota (Fàbrega & Vila, 2013). Furthermore, reactive oxygen species (ROS) produced in the gut in response to *Salmonella* react with thiosulfate present in the intestine to form tetrathionate. Tetrathionate can be utilised by *Salmonella* as an electron acceptor, providing a growth advantage for *Salmonella* in the anaerobic conditions of the intestinal mucosal layer (Fàbrega & Vila, 2013). Another advantage arises from the *Salmonella* virulence factor SopE, which induces the expression of nitric oxide synthase in the gut, leading to the production of NO. The reaction of NO with ROS leads ultimately to the formation of nitrate which, having a higher standard redox potential than tetrathionate, is then used by *Salmonella* as an anaerobic electron acceptor. This increased nitrate respiration leads to greater *Salmonella* growth, aiding *Salmonella* in outcompeting commensal microbiota. Ultimately *Salmonella* that survives these accumulative host-defences will encounter and adhere to the brush

border membrane of the epithelial cells of the gut (Prior et al, 2009; Levy *et al*, 2006).

1.2.2.2 Invasive Phase

Salmonella entry into the gastrointestinal epithelia appears to occur preferentially through adherence and entry into the microfold-cells (M cells) of the Peter's Patches (PP) in the intestinal epithelium (Prior et al, 2009; Fàbrega & Vila, 2013). It has also been observed that *Salmonella* can invade via normally non-phagocytic enterocytes (Fàbrega & Vila, 2013; Prior *et al*, 2009; Haque *et al*, 2004). Shortly after adhesion to the epithelial membrane *Salmonella* virulence factors interact with host cell signalling pathways to induce significant cytoskeletal rearrangement (Fàbrega & Vila, 2013). This results in membrane ruffling, leading to the internalisation of the bacteria into the cell and engulfment into a vesicle known as *Salmonella* containing vacuole (SCV) (Fàbrega & Vila, 2013). This intracellular compartment is distinct from the classical phagosome and its formation and maintenance is controlled by the bacterium itself, providing a niche in which *Salmonella* can survive and replicate (Fàbrega & Vila, 2013). Simultaneously, the host-immune system is alerted to the presence of the bacteria by Toll-like receptors (TLR) on host cells. These receptors recognize small molecular motifs present on bacterial cell surfaces known as pathogen-associated molecular patterns (PAMPs) (Prior *et al*, 2009; Mumy & McCormick, 2005). These PAMPs identify the bacteria to the host immune system as non-self and therefore stimulate an innate immune response against *Salmonella* (Prior *et al*, 2009). PAMPS include the lipopolysaccharide (LPS) that is part of the

Salmonella cell wall, and various secreted bacterial proteins (Prior *et al*, 2009). This leads to recruitment and transmigration of phagocytes into the intestinal lumen from the submucosa and stimulates the production of proinflammatory cytokines such as tumour necrosis factor alpha (TNF-a) and interleukin-8 (IL-8) (Fàbrega & Vila, 2013). Following bacterial internalisation into the SCV, *Salmonella* again interacts with host-cell signalling pathways to reform the apical epithelial brush border (Fàbrega & Vila, 2013). As *Salmonella* bacteria preferentially invade the intestinal epithelium by entry into M cells, they are directly transported to the lymphoid cells underlying PP (Fàbrega & Vila, 2013). The invading bacteria are engulfed by various phagocytes, including neutrophils and inflammatory monocytes, which are recruited from the blood as a result of inflammatory signals (Fàbrega & Vila, 2013). Systemic dissemination of *Salmonell*a is facilitated by infected phagocytes which migrate via the blood stream or lymphatic system to the liver and spleen (Fàbrega & Vila, 2013). *Salmonella* then multiply in these organs before re-entering the blood, known as secondary bacteriosis, and being transported back to the intestinal tract via the gall bladder. With Typhoid the secondary exposure of the PP to the bacteria causes damage which may result in ulceration, bleeding and necrosis, leading to perforation of the PP and in severe cases, death (Everest *et al*, 2001).

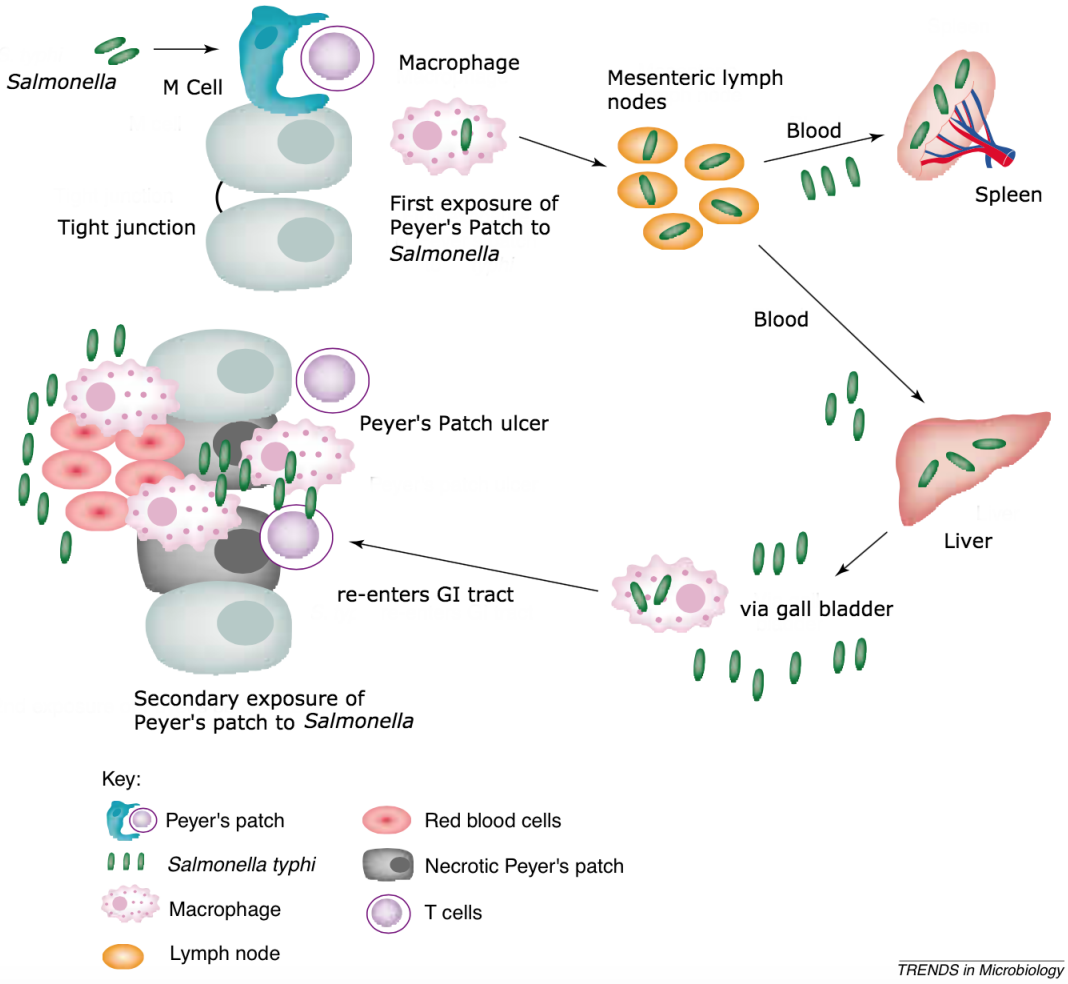


Figure 4: Systematic invasion of *Salmonella*. *Salmonella* breach the intestinal epithelium via M cells in Peyer's patches (PP) and are taken up by macrophages which transport them to the mesenteric lymph nodes. They then utilise these immune cells to migrate via the blood to the liver, spleen and bone marrow (not shown) where the bacteria multiply. *Salmonella* then re-enters the intestinal tract via the gall bladder, resulting in tissue damage to PP that may lead to ulceration and necrosis (Figure taken from Everest *et al*, 2001).

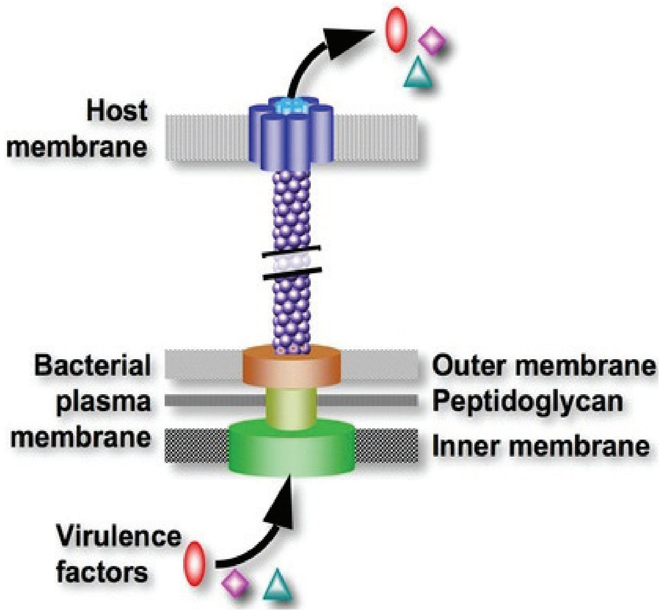
1.3 *Salmonella* Pathogenicity Islands

The ability of *Salmonella* to cause disease is dependent on its ability to invade non-phagocytic host cells, which in turn is largely reliant on a group of virulence genes known as *Salmonella* pathogenicity islands (SPI). There are currently five identified SPIs (SPI-1 to 5) known to be involved in *Salmonella* virulence. Together with other pathogenic components, including the pST plasmid-carried *spv* operon, various adhesions, flagella and essential genes involved in biofilm formation, SPIs are responsible for the virulence of *Salmonella* (Ellermeier & Slauch, 2007). SPI-1 is of particular note, being vital for the invasion of epithelial cells in the host intestinal lumen by arbitrating rearrangement of host cell actin cytoskeletons to facilitate internalization of the bacteria (Fàbrega & Vila, 2013). The importance of SPI-1 is demonstrated by SPI-1 mutants, which are unable to infect the host through the oral route and are incapable of *in vitro* invasion of enterocytes (Lucas & Lee, 2001).

The genes of the SPI-1 locus encode a type three secretion system (TTSS), a structure which resembles a hypodermic syringe and through which *Salmonella* translocates effector proteins into host cells (Srikanth *et al*, 2011; Prior *et al*, 2009) (Figure 5A). The *Salmonella* TTSS is composed of some 20-30 proteins, most of which have a structural role in the formation of the 'needle' (Kubori *et al*, 2000). The remaining are secreted effector proteins including, but not limited to, SipA, SipC, SopB, SopE and SopE2 (Agbor & McCormick, 2011). Whilst the large majority of secreted effectors involved in the invasion process are located within SPI-1, additional effectors appear to be encoded elsewhere on the chromosome (Fàbrega & Vila, 2013; Lostroh & Lee, 2001). The needle complex itself is encoded by the so-called *prg/org* and

inv/spa operons, whilst the *sic/sip* operon encodes the effector proteins. It is the pore-forming structure called the translocon, composed of SipBCD, which inserts itself into host cell membrane and 'injects' these effectors to the host cytosol. Together these proteins then function to alter cytoskeletal structure and induce membrane ruffling, leading to eventual engulfment of the *Salmonella* bacterium into the cell (Figure 5B). SopE, SopE2 and SigD (itself encoded on SPI-5) are largely responsible for the initiation of cytoskeletal remodelling. SopE acts as a guanidine exchange factor (GEF) leading to the activation of Cdc42 and Rac-1, two small host Rho GTPases which stimulate cell actin cytoskeletal modifications. SipA performs the role of stabilising the actin filaments and promotes the extension of outward membrane ruffles whilst SipC nucleates actin and initiates polymerization, both leading to alteration of the cytoskeletal structure (Fàbrega & Vila, 2013; Zhang *et al*, 2008). This alteration is further achieved by *Salmonella's* mediation of phosphoinosides, leading to the increased elasticity of the plasma membrane (Zhang *et al*, 2008). Following *Salmonella* invasion, an additional SPI-1 effector, SptP, performs as a GAP to inactivate Cdc42 and Rac1 and cause reformation of normal host-cell morphology (Zang *et al*, 2008). This process is not dissimilar to the process by which phagocytes engulf invading pathogens and results in the internalisation of *Salmonella* within the host cell (Pior *et al*, 2009).

A



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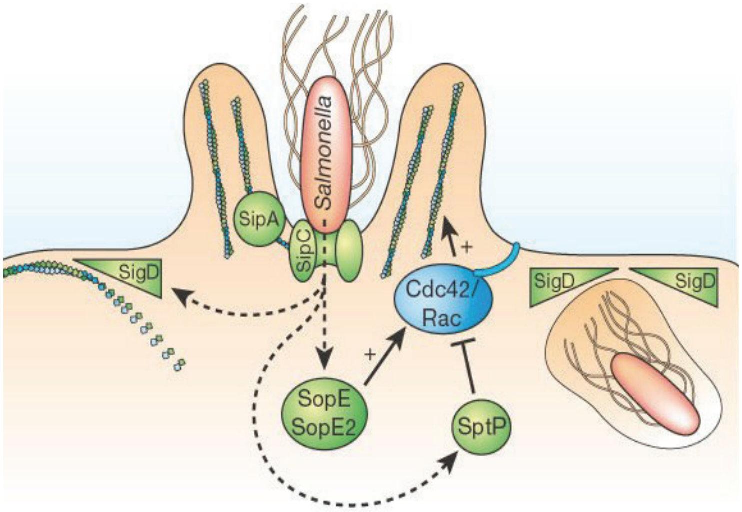


Figure 5: The TTSS and effector proteins of *Salmonella.* (A) The type three secretion system (TTSS) of *Salmonella* encoded by SPI-1. This cylindrical, needle-like structure allows secretion of *Salmonella* effector invasion proteins into the host cell cytosol. (B) Effector proteins of SPI-1 in *Salmonella* interact with host cell mechanisms to cause alteration of the cell actin cytoskeleton and induce membrane ruffling, leading to the eventual engulfment of the bacteria in the cell (Figures taken from Zang *et al*, 2008).

1.3.1 Control of SPI-1

Induction of SPI-1 and its components is controlled by the master regulator and activator, Hyperinvasion Locus protein A (*HilA),* which is also located on the SPI-1 locus. HilA is a member of the OmpR/ToxR family and its up-regulation is vital for the expression of SPI-1 genes and therefore for *Salmonella* invasion to occur (Lucas & Lee *et al*, 2001; Baxter & Jones, 2015). Activation of HilA is controlled by a multi-component feed forward regulatory loop consisting of three AraC-like regulators, HilD, HilC and RtsA. All three of these regulators are capable of independently activating HilA expression (Srikanth *et al*, 2011) (Figure 6). Both HilD and HilC are required for *hilA* induction, even when multiple repressors are absent (Boddicker *et al*, 2002). These transcriptional activators are also located within SPI-1 and bind directly to promoter sequences upstream of *hilA* to induce its expression (Baxter & Jones, 2015). RtsA itself is encoded outside of SPI-1, though it works in concert with HilD and HilC in the feed-forward loop. HilD is also thought to activate HilA and rtsA expression by removing the repression of H-NS, a nucleoid-associated protein that inhibits the expression of SPI-1 (Martinez *et al*, 2014). *HilA* expression is negatively modulated by various additional factors, the most crucial of which is HilE. HilE exerts its effect by interacting with HilD and preventing it from activating *hilA*, therefore leading to SPI-1 repression (Baxter & Jones, 2015).

Following entry into the intestinal lumen *Salmonella* encounters the microenvironment of the intestinal epithelial cells, namely high pH, low oxygen and high osmolality. In these conditions, repression is completely removed as *Salmonella* enters the stationary phase and the expression of *hilA* is

accordingly induced (Queiroz *et al*, 2011). These individual environmental conditions are thought to act themselves as cues for the induction of *HilA*, leading in turn to the up regulation of SPI-1 and the secretion of effector proteins for host-cell invasion (Srikanth *et al*, 2011).

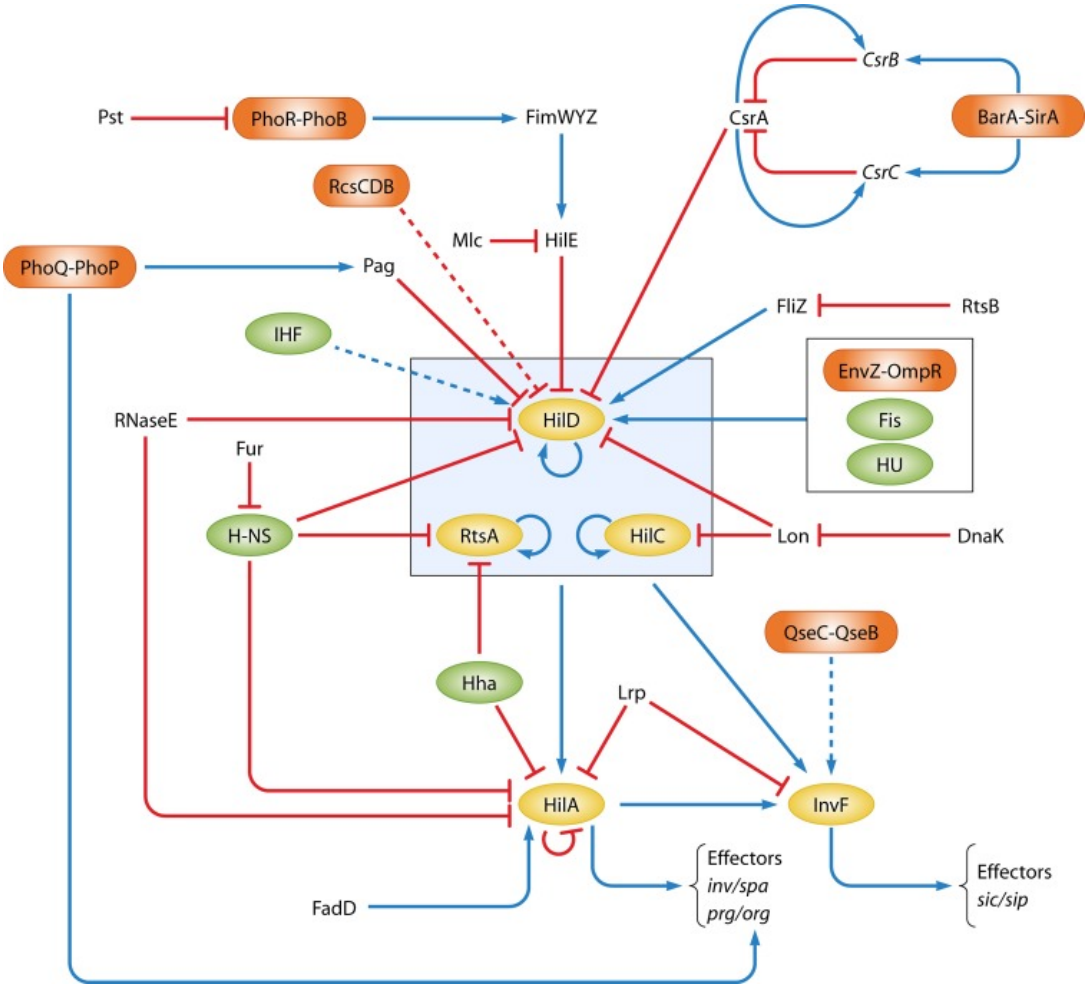


Figure 6: Control of the HilA transcriptional activator. *HilA* is a master regulator and transcriptional activator of SPI-1 genes in *Salmonella* and is itself controlled by a complex feed-forward loop. It is activated by three AraC-like activators, HilD, RtsA and HilC and is repressed by the transcriptional repressor HilE and the global repressor H-NS (Figure taken from Fàbrega & Vila, 2013).

1.4 *Salmonella* and Reactive Nitrogen Species (RNS)

Reactive Nitrogen Species (RNS) are the oxidized forms or adducts of nitrogenous products originally formed by nitric oxide synthases (Nathan & Shiloh, 2000). Various RNS include nitric oxide (NO), nitrogen dioxide ($NO_2^{\cdot}$), dinitrogen trioxide (N_2O_3) and peroxynitrite ($ONOO^-$), all of which are encountered in physiological environments (Koskenkorva-Frank *et al*, 2013; Nathan & Shiloh, 2000). RNS are capable of damaging lipids, proteins and DNA and are therefore under strict physiological regulation (Koskenkorva-Frank *et al*, 2013).

Many mammalian cell types produce RNS, often in response to inflammatory stimuli, the most notable of these being macrophages. Also produced by macrophages are reactive oxygen species (ROS), such as superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\cdot}$), as by-products during mitochondrial electron transport or by the oxidation of various metabolites. The dismutation of $O_2^{\cdot -}$ forms H_2O_2, the latter of which can oxidize thiol groups and react with Fe(II) and Fe(III) to form other damaging compounds such as hydroxyl radicals ($OH^{\cdot}$) and hydroxide (OH^-). Furthermore, both hydrogen peroxide and superoxide anions are capable of damaging iron-sulphur ([Fe-S]) clusters (Arkenberg, 2013).

NO can react with such ROS to form other RNS, for instance the production of $O_2^{\cdot -}$ and NO in macrophages is nearly equimolar, and the reaction of these two products results in the generation of $ONOO^-$ (Nathan & Shiloh, 2000). Other toxic intermediates produced by the reaction of RNS with ROS and their interactions are described in Figure 7.

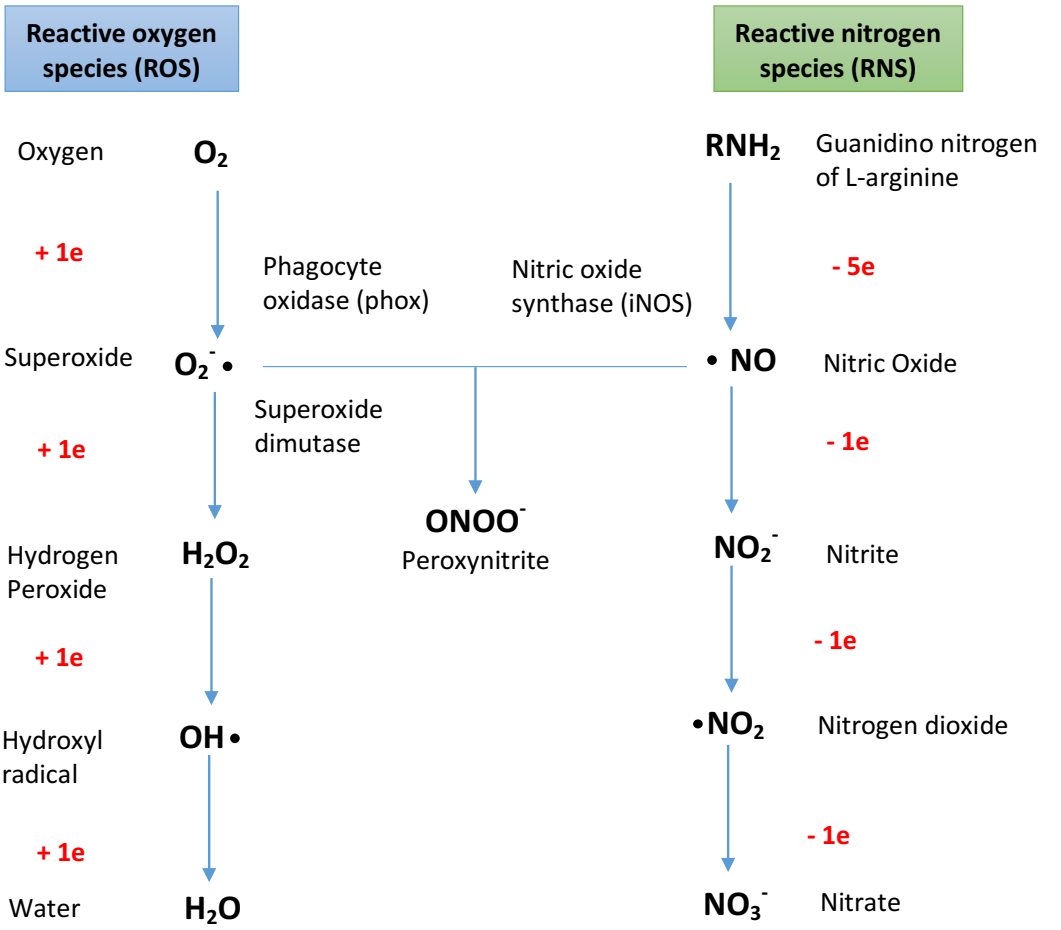


Figure 7: Production and interactions of Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS). ROS and RNS are produced in mammalian cells by phagocyte oxidase (phox), also known as NADPH oxidase, and Nitric oxide synthase (iNOS) respectively. NO may react with superoxide ($O_2^{\cdot -}$) to form peroxynitrite, a powerful oxidant and nitrating agent. NO may also be oxidized to nitrite (NO_2^-), which then can be oxidized to nitrogen dioxide (NO_2) and this product may be oxidized further to nitrate (NO_3^-). ROS can produce superoxide ($O_2^{\cdot -}$) which may under dismutation catalysed by superoxide dismutase (SOD) to become hydrogen peroxide (H_2O_2). Dissociation of H_2O_2 produces hydroxyl radicals ($OH^{\cdot}$) which can react with other volatile organic compounds to lose a hydrogen and form water (H_2O) (Figure adapted from Fang, 2004).

NO a colourless gas containing an odd number of electrons, making it a radical with high reactivity (Kelm, 1999). It is produced at low concentrations during normal cellular metabolism and functions as a molecular messenger in eukaryotic cellular signalling (Koskenkorva-Frank *et al*, 2013; Park *et al*, 2011; Pacher *et al*, 2007). However, NO is also a potent cytotoxin lethal to most pathogens and thus also acts as an effector of the innate immune system (Arkenberg *et al*, 2011). As NO is a small, lipophilic free radical it is capable of diffusing freely in the cell and across bacterial membranes to exert bactericidal and bacteriostatic effects (Park *et al*, 2011; Atkan, 2004). Mammals unable to produce antimicrobial NO are significantly more susceptible to viral, fungal, parasitic and bacterial invasion (Richardson *et al*, 2011). Notably, NO has been shown to be a critical component for controlling *S.* Typhimurium infection in murine models (Richardson *et al*, 2011). For instance, mice with abrogated *iNOS* alleles are more susceptible to infection with *S.* Typhimurium than control mice (Mastroeni *et al*, 2000). A 2002 study demonstrated that iNOS-deficient mice infected with an avirulent strain of *Salmonella* perished after 6 days due to severe septicaemia, which WT mice did not contract (Alam, 2002). Despite this, understanding of how NO and other RNS exert their bacteriostatic effects is limited and mechanisms of such action remains to be fully elucidated (Richardson *et al*, 2011; Park *et al*, 2011). Metabolic enzymes with essential redox centres comprised of protein thiols, Fe-S clusters or heme groups likely represent the major targets of NO-mediated growth inhibition (Richardson *et al*, 2011; Cooper, 1999). Such cofactors are vital for various essential cellular processes such as electron transfer, nitrogen fixation and gene regulation. For example, FNR, Fur and

NsrR are all transcriptional regulators containing Fe-S clusters which may undergo nitrosylation, consequently inhibiting their DNA binding (Isabella *et al*, 2009). NO has a high affinity for the terminal quinol cytochrome oxidases of the electron transport chain and can inhibit the ability of *Salmonella* to reduce O_2 to H_2O, resulting in respiratory arrest in nitrosatively stressed cells (Henard & Vazquez-Torres, 2011, Richardson, 2011). Many enzymes involved in glycolysis, fatty acid metabolism, glutamate biosynthesis and pyruvate catabolism consistently undergo thiol modification during nitrosative stress. Enzymes with [Fe-S] clusters are involved within the citric acid cycle and branched-chain amino acid biosynthesis and as a result of NO inhibiting such enzymes, *Salmonella* is unable to synthesise methionine or lysine during nitrosative stress (Richardson *et al*, 2011). NO can also react with the tyrosyl radical in the active site of ribonucleotide reductase. The nitrosylation of ribonucleotide reductase disrupts the formation of deoxyribonucleotides needed for repair and synthesis of DNA and may likely contribute to bacteriostatic effects of NO against *Salmonella* (Henard & Vázquez-Torres, 2011). The indirect effects of NO on biological targets are mediated through the RNS generated from the reaction of NO with other molecules. NO is capable of reacting with superoxide anions ($O_2^{\cdot-}$) or molecular oxygen (O_2) in macrophages to form dinitrogen trioxide (N_2O_3), which is a potent oxidizing agent. Also in macrophages NO and the superoxide anion can combine to form highly reactive peroxynitrite ($ONOO^-$), which is a powerful oxidant and nitrating agent and damages a wide array of molecules in cells including SH-groups, lipids, DNA and proteins (Szabó, 2003; Prior *et al*, 2009). It can also react with carbon dioxide (CO_2) to form nitrosoperoxocarbonate ($ONOOCO_2^-$)

which is highly reactive. Through oxidation or nitration mechanisms peroxynitrite can irreversibly inhibit mitochondrial respiration and damage a variety of mitochondrial components. It can therefore trigger cellular responses ranging from subtle modulations of cell signalling to overwhelming oxidative tissue damage, committing cells to necrosis or apoptosis (Virág, 2002).

NO is therefore a potent antimicrobial component of the innate immune system and plays a considerable role in the control of *Salmonella* infection.

1.4.1 Nitric Oxide in the Gastrointestinal Tract

As previously discussed, *Salmonella* is exposed to a range of RNS in the stomach and gut which act as a defence against infection (Henard & Vazquez-Torres, 2011). The majority of RNS present in the gastric lumen are from exogenous dietary sources. Nitrate is obtained from vegetables such as beets, celery and leafy greens in particular, whilst cured and processed meats can be a source of nitrite, which is added as a preservative (Prior *et al*, 2009). Concentrations of NO in the gastrointestinal tract therefore fluctuate from some 100μM to 500μM depending on dietary consumption of nitrate and nitrite (Prior *et al*, 2009; Henard & Vazquez-Torres, 2011). Commensal microbiota in the mouth first reduce dietary nitrate to nitrite, which is then protonated in the stomach to nitrous acid (HNO_2). The disproportionation of HNO_2 forms various RNS including NO, NO_2 and N_2O_3 (Henard & Vazquez-Torres, 2011). At low pH conditions, such as those encountered in the stomach, RNS have direct bactericidal effect against *Salmonella*. At slightly higher pH RNS can have indirect antimicrobial activity, such as interfering with

the acid tolerance response of *Salmonella* that allows it to survive strongly acidic conditions (Henard & Vazquez-Torres, 2011).

1.4.2 Nitric Oxide in Macrophages

Macrophages are mononuclear phagocytic leukocytes which have key roles in both innate and adaptive immunity (Prior *et al*, 2009). Some may be located in specific tissues, such as Kupffer cells in the liver, whilst others circulate in the blood plasma and are then recruited to sites of infection (Prior *et al*, 2009). They are an essential part of the immune response to invading pathogens and their ability to produce NO is vital for controlling *Salmonella* infection (Prior *et al*, 2009). NO is produced in the body by nitric oxide synthases (NOSs) of which there are three known isoforms (Aktan, 2004). These include epithelial nitric oxide synthase (eNOS), neuronal nitric oxide synthase (nNos) and inducible nitric oxide synthase (iNOS) (Aktan, 2004). In macrophages NO is produced by iNOS which catalyses the conversion of L-arginine and molecular oxygen to L-citrulline and NO as part of the innate immune response (Aktan, 2004). In contrast to eNOS and nNOS, iNOS is able to generate NO without the need for elevated intracellular calcium (Ca^{2+}) (Chakravortty & Hensel, 2003). As high levels of NO are cytotoxic to host-cells as well as pathogens, iNOS activation is highly regulated in order to prevent overproduction of NO (Atkan, 2004). Unlike eNOS and nNOS which are constitutively present and active in resting cells, expression of iNOS is low in macrophages before its transcription is up-regulated by immunostimulatory cytokines and PAMPS (Cherayil & Antos, 2001). The lipopolysaccharide (LPS) component of the cell wall of *Salmonella* is one such molecule. LPS is

bound by the LPS-binding protein (LBP) which transports it to a receptor with high affinity for the LPS molecule, known as CD14. This LPS-CD14 complex interacts with TLR4 and associated small extracellular MD-2 protein to activate an intracellular signalling cascade via adaptors such as IRAK and MyD88. Ultimately this results in the activation of the NFκβ signalling pathway, which activates transcription of iNOS in the nucleus (Lowenstein & Padalko, 2004). Inflammatory cytokines such as tumour necrosis factor alpha (TNF-α) and interleukin 1β (IL-β), which are produced by infected host cells, and Interferon-γ (IFN-γ), produced by activated immune cells, can also activate iNOS expression (Cherayil *et al*, 2000; Lowenstein & Padalko, 2004). IFN-gamma signalling results in dimerization of JAK proteins and subsequently leads to phosphorylation and translocation of a STAT protein to the nucleus to increase expression of IRF-1 transcription factor. This transcription factor binds to the iNOS promoter in the nucleus and causes up-regulation of iNOS gene expression (Prior *et al*, 2009). Similarly, SPI-1 effectors can stimulate the formation of inflammasomes and activation of IL-Iβ and IL-18. Caspase-1 has also been shown to be able to induce the expression of iNOS and lead to production of NO (Henard & Vazquez-Torres, 2011; Buzzo *et al*, 2010).

As well as NO, macrophages also produce ROS, enabling them to bombard *Salmonella* with so-called respiratory bursts. When activated a multicomponent nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidase is responsible for catalysing the reduction of molecular oxygen to superoxide. This radical is then able to combine with other oxygen species for form more toxic ROS such as hydrogen peroxide and hydroxyl

radicals (Forman & Torres, 2002; Vazques-Torres & Fang, 2001). The respiratory burst plays a powerful role in innate defence; those individuals lacking functional NADPH oxidase are prone to recurring and severe bacterial and fungal infections (Fang & Vaquez-Torres, 2002). The NADPH oxidase in phagocytes is NADPH oxidase 2, or NOX2, and is assembled at the plasma membrane. In resting cells, the membrane-bound and cytosolic components of the enzyme are separated by vesicles and only come together when the enzyme is activated by various stimuli. Such stimuli include arachidonic acid, phospholipases A and D and protein kinase C. Activation leads to cytosolic components migrating to the plasma membrane to assemble to form the active NADPH oxidase enzymatic complex (Vazquez-Torres & Fang, 2001). Together with iNOS, the respective oxidative and nitrosative bursts produced by these enzymes play a critical role in the killing of *Salmonella* inside macrophages.

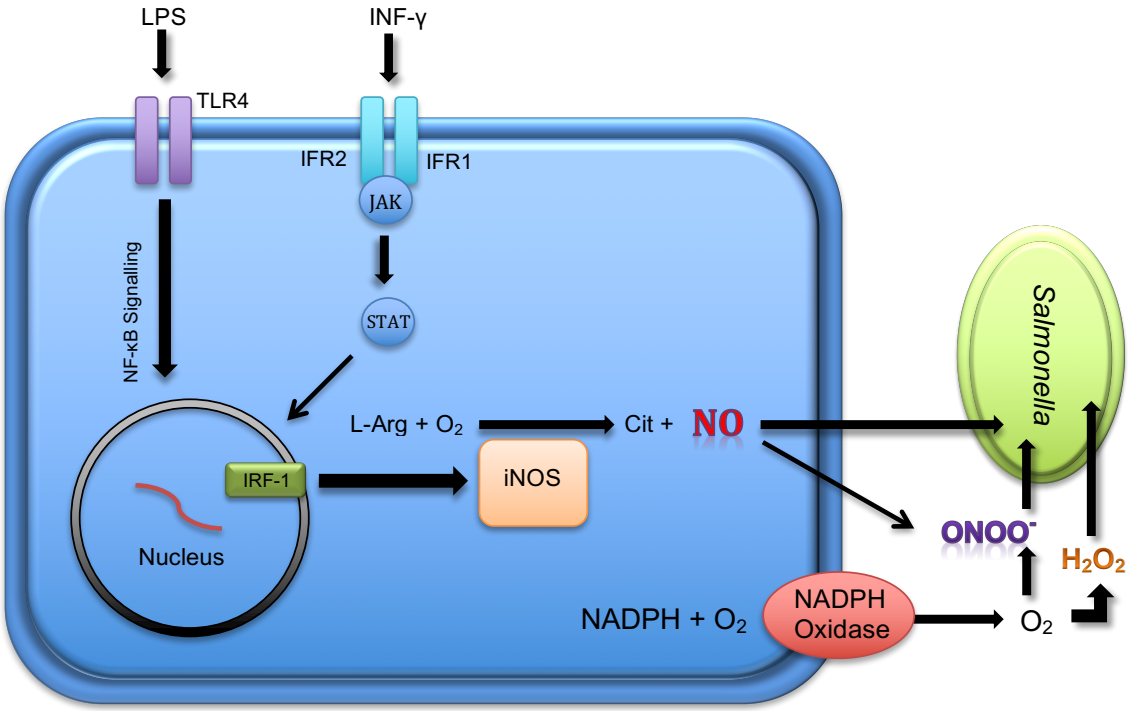


Figure 8: Activation of iNOS and production of NO inside macrophages. PAMPS such as LPS and immunostimmulatory cytokines such as Interferon-γ (INF-γ) produced by activated immune cells are capable of activating iNOS expression. IFN-γ signalling leads to JAK-STAT signalling which ultimately increases the expression of the IRF-1 transcription factor. IFR-1 binds to the iNOS gene promoter region in the nucleus and causes up-regulation of iNOS gene expression. The LPS component of *Salmonella* is detected by TLR4 which induces an NF-κB pathway leading to activation of iNOS transcription in the nucleus. Once active, iNOS catalyses the reaction of L-arginine and molecular oxygen to produce L-citrulline and NO. NO may exert bacteriostatic effects on *Salmonella* and it may also join with superoxide produced by NADPH oxidase to form the highly oxidative and nitrosative peroxynitrate ($ONOO^-$). Superoxide may also spontaneously form hydrogen peroxide (H_2O_2) which damages *Salmonella* through oxidative stress, or may undergo further reactions to generate other ROS. (Figure adapted from Lowenstein & Padalko, 2004.)

1.4.3 Nitric Oxide in *Salmonella* Nitrate Catabolism

Salmonella is a facultative anaerobe, meaning that whilst it preferentially utilizes oxygen as an energy source, in low oxygen conditions it is able to switch to nitrate metabolism, a process known as denitrification (Arkenberg *et al*, 2011). This process involves the use of nitrate reductase systems that will be discussed later. Whilst in most soil bacteria nitrate is ultimately converted to N_2, both *Salmonella* and *E. coli* undergo truncated denitrification, where nitrate is converted into nitrous oxide (N_2O) via nitrite and NO (Arkenberg *et al*, 2011). This is due to it lacking the nitrous oxide reductase (NosZ), which converts N_2O into N_2. The result being that when nitrate is used as an alternative electron acceptor NO is produced a toxic intermediate which *Salmonella* must be able to detoxify to survive.

1.5 Nitric Oxide Detoxification

Salmonella survival relies on the bacteria having some mechanism by which to detoxify the endogenous and exogenous NO it encounters. Three metalloenzymes are well-characterized for their involvement in NO detoxification in *Salmonella*, including the cytochrome *c* nitrite reductase (*nrfA*), flavorubredoxin (*norV)* and flavohemoglobin (*hmpA*). These three enzymes operate under different oxygen conditions (Prior *et al*, 2009) (Figure 9).

1.5.1 Cytochrome c nitrite reductase

Cytochrome c nitrite reductase (NrfA) is an anaerobic respiratory enzyme which is expressed in the presence of nitrate and only under anoxic or micro-

oxic conditions. When active it is able to reduce either NO_2 or NO to ammonium (NH_4) (Prior *et al*, 2009). NrfA is a multihaem, periplasmic protein which appears to be most active as a NO reductase in acidic conditions (van Wonderen *et al*, 2008). It might therefore play a role in NO detoxification in the micro-oxic and very acidic contains of the stomach, where there is a high disproportionation of nitrate to NO (Prior *et al*, 2009). However, NrfA does not seem to be required for *Salmonella* survival in mice (Prior *et al*, 2009).

1.5.2 Flavorubredoxin

Flavorubredoxin (*NorV*) is an oxygen sensitive NO reductase with an associated oxidoreductase (*NorW)* (Mills *et al*, 2005). It is active under anaerobic or low-oxygen conditions where it reduces NO to nitrous oxide (N_2O). The transcriptional activator NorR senses NO levels and induces transcription of *norV* (Prior *et al,* 2009). It has been well studied in both *E. coli* and *Salmonella* and does not appear to be necessary for *E. coli* survival in murine macrophages or for *Salmonella* survival in mice (Prior *et al*, 2009).

1.5.3 Flavohemoglobin

The flavohemoglobin (HmpA) consists of an N-terminal heme group and C-terminal reductase and is active under both anaerobic and aerobic conditions. In aerobic environments HmpA oxidises NO to NO_3, whilst in anaerobic environments it reduces NO to N_2O. Though its precise significance under anaerobic conditions remains to be elucidated, it is known to be the major enzyme responsible for aerobic NO metabolism (Prior *et al*, 2009; Bang *et al*, 2006). The expression of *hmpA* is induced by NO and repressed by the

presence of intracellular iron (Prior *et al*, 2009). It is also repressed in the absence of NO, by the nitric oxide sensitive repressor (NsrR). NsrR is a member of the Rrf2 family of transcriptional factors and contains an iron-sulfur (Fe-S) cluster which is necessary for NsrR to bind DNA and repress *hmpA* expression. NO is capable of nitrosylation of the Fe-S cluster, which abrogates the ability of NsrR to bind DNA and therefore lifts repression of *hmpA* (Karlinsey *et al*, 2012). The NsrR regulon is now known to include *hcp-hcr, yeaR-yoaG, ygbA, ytfE* and *STM1808*, as well as *hmpA* (Karlinsey *et al*, 2012). Studies have shown that *hmpA* expression is highly induced in macrophages, suggesting that it plays a vital role in detoxification of NO produced by iNOS (Eriksson *et al*, 2003). By contrast, it is not induced in epithelial cells, where *Salmonella* is not exposed to either oxidative or nitrosative stress (Hautefort *et al*, 2008).

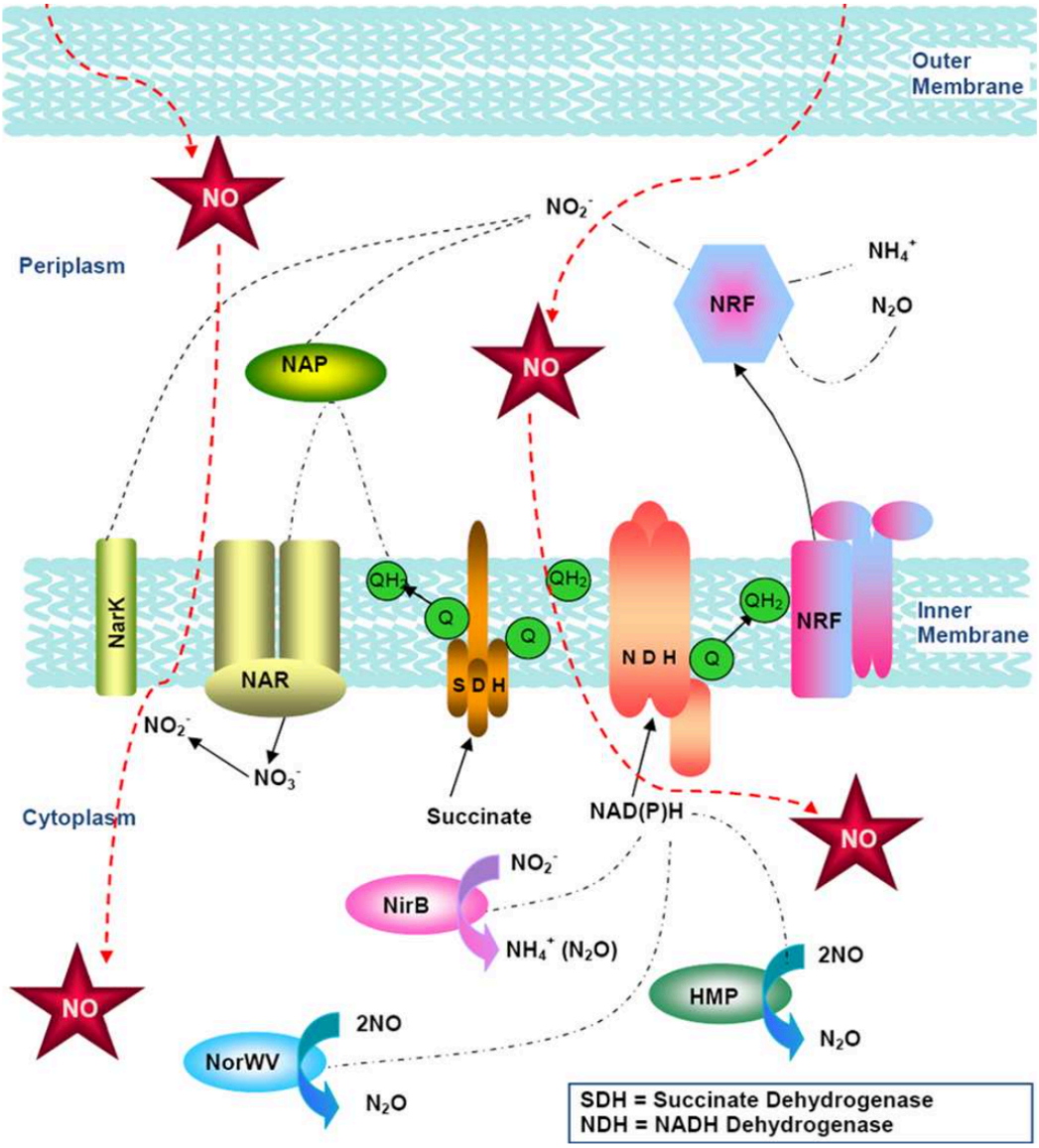


Figure 9: Pathways of Nitrate Catabolism and Nitric Oxide detoxification in *Salmonella.* NO is produced endogenously as a by-product of *Salmonella* denitrification, where *Salmonella* utilises nitrate as an alterative electron acceptor under anaerobic conditions. As part of this process two distinct nitrate reductase systems are utilised, the membrane bound NarGHJI and the periplasmic NapA. In the cytoplasm NarG reduces nitrate to nitrite, which is then further reduced to NH_4 by the sirohaem-containing nitirite reductase (NirB). In the periplasm nitrate is reduced to nitrite by NapA, and further reduced to NH_4 by the cytochrome *c* nitrite reductase (NrfA). Detoxification of both endogenously and exogenously produced NO is carried out by three main enzymes; flavohaemoglobin (HmpA), flavorubedoxin and associated oxidoreductase (NorVW) and Cytochrome c nitrite reductase (NrfA.) NorVW and NrfA are only active under anaerobic or micro-oxic conditions. HmpA has only a minor role in NO detoxification under anoxic conditions, but is the crucial enzyme when oxygen is present. NrfA reduces nitrite or NO to NH_4, NorV reduces NO to N_2O and HmpA oxidizes NO to NO_3 (oxic conditions) or reduces NO to N_2O (anoxic conditions). (Figure taken from Prior *et al*, 2009.)

1.6 Nitrate Reductase Systems

As previously discussed, *Salmonella* is capable of using nitrate (NO_3) as an alternative electron acceptor when oxygen is unavailable. In order to accomplish this reaction *Salmonella* synthesizes two biochemically distinct nitrate reductases, a membrane-bound system (NarGHI) and a periplasmic system (NapA) (Rowley *et al,* 2012). These two systems have corresponding nitrite reductases which further reduce the NO_2 to NH_4 in the cytoplasm and periplasm respectively. The NarG system was the first nitrate reductase system identified in *Salmonella* and is expressed under high nitrate concentrations (Prior *et al*, 2009). NarG reduces NO_3 to NO_2 in the cytoplasm (reaction 1) and this NO_2 is further reduced to NH_4 by a sirohaem-containing nitrite reductase (NirB) (reaction 2).

Reaction 1: $NO^-_3 + 2e^- + 2H^+ \rightarrow NO^-_2 + H_2O$

Reaction 2: $NO^-_2 + 6e^- + 8H^+ \rightarrow NH^+_4 + 2H_2O$

NapABC is the periplasmic nitrate reductase encoded by the NapFDAGHBC (NapF) operon which was more recently identified. NapA is the catalytic subunit which reduces NO_3 to NO_2 in the periplasm (Dow *et al*, 2014) (reaction 1). The periplasmic cytochrome *c* nitrite reductase (NrfA) then reduces this NO_2 to NH_4 (reaction 2) (Rowley *et al*, 2012). This process, which produces extracellular NH_4, is known as dissimlatory nitrate reduction to ammonium (DNRA) (Rowley *et al*, 2012). NO is produced as a toxic intermediate to these reactions and must be detoxified by the mechanisms described above. Because of its location in the periplasm, it is possible that

NapA performs the initial step in nitrate reduction (Rowley *et al*, 2012). However, in high nitrate conditions NarG acts as the main nitrate reductase, for instance when active in *E. coli* under high nitrate conditions it performs 98% of nitrate reductase activity (Rowley *et al,* 2009). NapA, which is repressed in the presence of oxygen, may conversely be the main nitrate reductase under nitrate-limited conditions. Whilst NarG is energy efficient, it has low-affinity for nitrate, whereas NapA has a high affinity for nitrate though it is less energy efficient. Therefore, when nitrate is scarce, NapA provides a higher affinity, though more poorly-coupled pathway for nitrate reduction (Rowley *et al*, 2012).

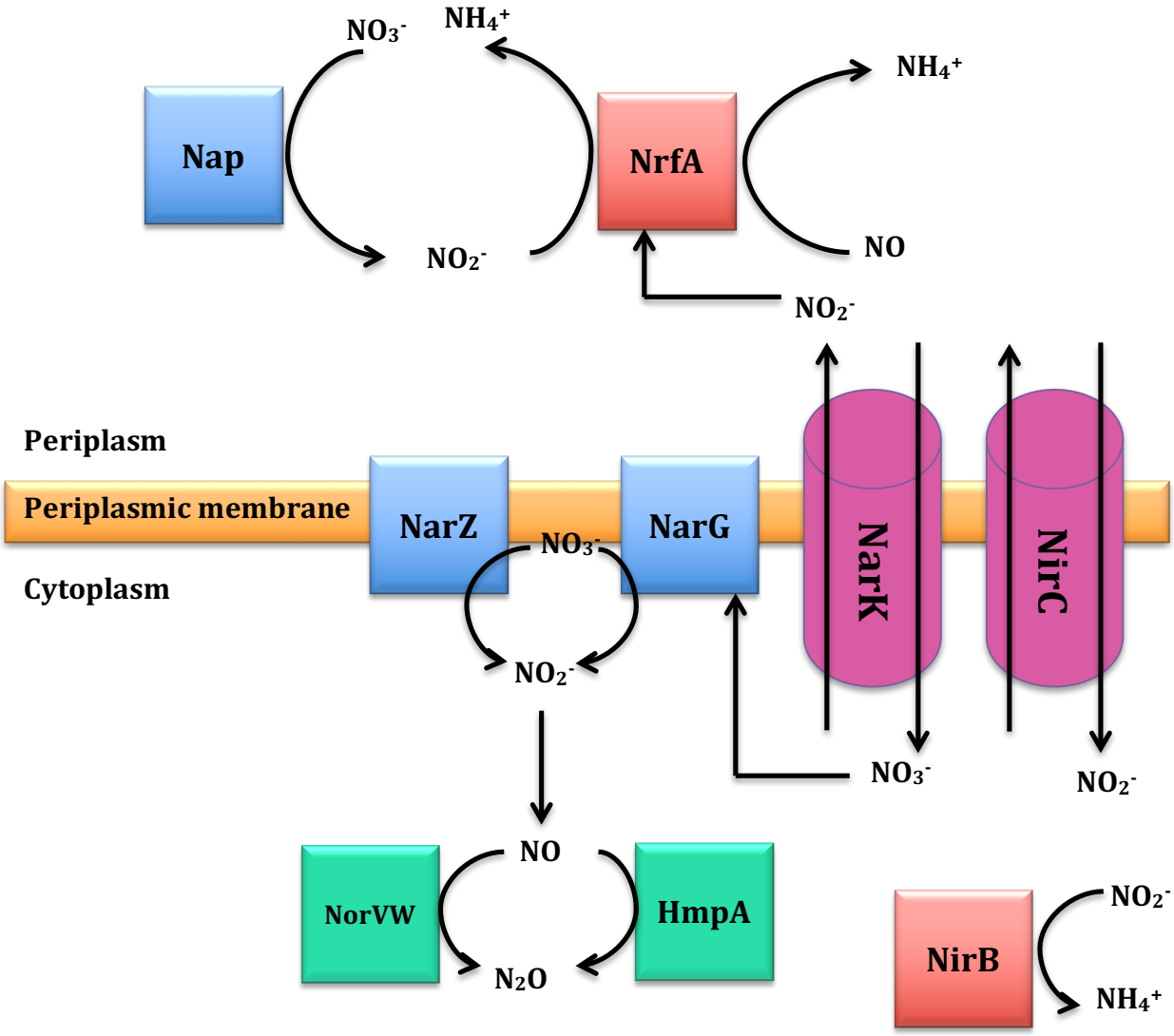


Figure 10: *Salmonella* denitrification enzymes and pathways of nitrate reduction. *Salmonella* is capable of utilising NO_3 as an alternative electron acceptor in a process called truncated denitrification by which it is ultimately reduced to N_2O. This process utilises either of two nitrate reductases systems, NarGHI (membrane-bound) or NapA (periplasmic) depending on nitrate conditions. The NarZYWV system is thought to be an isozyme of NarG expressed under aerobic growth, whilst NarG is expressed and predominately active under anaerobic conditions. Antiporters such as NarK and NirC transport NO_3, NO_2 and protons across the periplasmic membrane. In the cytoplasm NarG reduces NO_3 to NO_2 that is then reduced to NH_4 by NirB. Conversely, Nap in converts NO_3 to NO_2 in the periplasm, and the periplasmic cytochrome *c* nitrite reductase (NrfA) then reduces this NO_2 to NH_4. The reduction of this NO_3 by nitrate reductases such as NarG, it's isozyme, NarZ or NapA and their associated nitrite reductases provides energy for the bacterial cell and produces endogenous NO as a by product. NarG appears to be the main nitrate reductase system active under high nitrate conditions, whilst NapA may become predominately active under nitrate-limited conditions. (Figure adapted from Prior *et al*, 2009.)

1.7 Context and Aims

Members of the *Salmonella* genus are successful enteric pathogens that cause gastroenteritis and Typhoid Fever in humans. *Salmonella* infections are thought to be associated with some 1 million deaths per year worldwide and therefore are a significant burden on global health (Prior *et al*, 2009). Currently, two typhoid vaccines are internationally available, one of these being Ty21a, a live, oral typhoid vaccine consisting of an attenuated strain of *S.* Typhi. The other is a component vaccine formed from the purified capsular polysaccharide *S.* Typhi Vi antigen (Cheminay & Hensel, 2008; Thiem *et al*, 2011). Both are currently licensed for use against Typhoid and have been shown to successfully immunise against Typhoid fever (Garmory *et al,* 2002). However, these vaccines have limited efficiency and require frequent boosters (Anwar *et al*, 2014). Due to their shortfalls, these vaccines are ineffective at controlling Typhoid in regions where the disease is endemic and instead are mostly utilized as 'Traveller's Vaccines' for individuals from predominately high-income countries (Anwar *et al*, 2014). An efficient vaccine that controls the disease in Typhoid epidemic countries is, as yet, non-existent, and greatly needed (Anwar, *et al*, 2014). A further worrying issue is the development of antibiotic resistance in *Salmonella*, with genetic and genomic evolution leading to increasingly virulent and antibiotic-resistance strains (Onwuezobe *et* al, 2012; Hurley *et al*, 2014). Since the 1970s *S.* Typhi strains have shown resistance to ampicillin, choramphenicol and co-trimoxazole; three primary antibiotics (Wong *et al*, 2015). Furthermore, resistance to fluoroquinolone, used as an alternative since the 1990s, has been described in various parts of Asia (Bhutta *et al*, 2006). Moreover, this multidrug resistant typhoid appears to

result in more severe manifestations of the disease, with higher rates of complications and fatalities (Bhutta *et al*, 1996). A recent study in *Nature genetics* identified a MDR *S.* Typhi clade of *Salmonella*, H58, present in Asia and Africa, which is outcompeting the antibiotic-sensitive *S.* Typhi strain population. The study claims H58, ensuing from the overuse of antibiotics, appears to be causing MDR outbreaks of Typhoid in previously unaffected areas and identify it as a threat to global health (Wong *et al*, 2015). There have also been reports of NTS resistance to fluroquinolones, third-generation cephalosporins and possibly also carbapenem, particularly after the increase in the use of antibiotics for treating NTS in livestock (Hurley *et al,* 2014; Onwuezobe *et al*, 2012; Tatavarthy *et al*, 2014). Within the last century multi-drug resistant strains of Typhimurium have emerged and been associated with higher mortality and morbidity rates (Onwuezobe *et al*, 2012). In fact, more than 55% of Typhimurium isolates show multidrug resistance and strains which are resistant to ampicillin, chloramphenicol, streptomycin-spectinomycin, sulphonamides and tetracyclines have been reported in Spain and the UK and are also likely present in other European Countries (Kirkpatrick *et al*, 2005; Fàbrega & Vila, 2013; Weill *et al*, 2006; Cloeckert & Schwarz, 2001). A further worrying development has been the ermergence of an invasive-NTS strain of *Salmonella* (iNTS) in Africa, ST13, which also shows multidrug-resistance (Morpeth *et al,* 2009; Feasey *et al*, 2012). Currently there are no vaccines for NTS (Wick, 2011).

It is vital that new therapeutic targets are discovered in *Salmonella* in order to facilitate the creation of a new generation of effective antimicrobials and vaccines. Necessary for this is a thorough understanding of *Salmonella*

physiology and pathogenic mechanisms. Building on preliminary data from the Rowley lab, this study aims to characterize and investigate various genes, which may potentially be involved in the detoxification of NO in *Salmonella* and therefore aid *Salmonella* survival in the host. A further aim of this research is to determine whether the transcriptional activator HilA regulates the Nap nitrate reductase of *Salmonella*. This could have significant implications if Nap is otherwise involved in *Salmonella* colonisation and invasion of the host intestine.

CHAPTER 2: MATERIALS AND METHODS

2 Materials and Methods

2.1 Materials

All chemicals and reagents were obtained from Sigma Aldrich (UK) or Fisher Scientific (UK) unless otherwise specified. Where necessary solutions and media were made using dH_2O, exempting those used for RNA work, which were prepared using nuclease and protease-free molecular biology grade water (Sigma, W4502). This will hereon be referred to as 'Sigma Water'.

2.2 Bacterial Strains and Plasmids

The bacterial strain studied in this work was the isogenic, parental strain *Salmonella enterica* serovar Typhimurium SL1344 described by Hoiseth & Stocker, 1981. All mutagenic strains utilised in this study derive from this wild-type (WT) strain. Table 1 lists all bacterial strains used in this study, all plasmids used may be found in Table 2.

Table 1: Bacterial strains used

Strain	Genotype	Reference
SL1344	S.Typhimurium WT, His-	Hoiseth & Stocker, 1981
ΔSTM1250	SL1344 Δ*STM1250*::kan	This study
ΔSTM1250	SL1344 Δ*STM1250*::cat	This study
ΔSTM4552	SL1344 ΔSTM4552::kan	This study
ΔSTM4552	SL1344 ΔSTM4552::cat	This study
ΔSTM1586	SL1344 ΔSTM1586::kan	This study
ΔSTM3362	SL1344 Δ*STM3362*::kan	Arkenberg
ΔycfR	SL1344 Δ*ycfR*::kan	Arkenberg
ΔycfR	SL1344 Δ*ycfR*::cat	This study
ΔybiJ	SL1344 Δ*ybiJ*::kan	Arkenberg
ΔSTM3362 ΔycfR	SL1344 ΔSTM3362::kan, ΔycfR::cat	This study
ΔSTM3362- ΔycfR ΔybiJ	SL1344 ΔSTM3362::kan, ΔycfR::cat, ΔybiJ::kan	This study
ΔIbpA ΔIbpB	SL1344 ΔIbpA ΔIbpB::kan	This study
ΔSTM1250 ΔSTM251	SL1344 Δ*STM1250*::kan, Δ*STM1251*::kan	This study
ΔSTM1250 ΔSTM1251	SL1344 Δ*STM1250*::cat, Δ*STM1251*::cat	This study
ΔSTM1250 ΔSTM121 ΔIbpA ΔIbpB	SL1344 ΔIbpA ΔIbpB::kan, Δ*STM1250*::cm, Δ*STM1251*::cm	This study
ΔSL1344	SL1344:pMP220	Prior, 2011
ΔSL1344	SL1344Δ*hilA*:pMP220	Prior, 2011
ΔSL1344	SL1344:pMP220:*napF*	Prior, 2011
ΔSL1344	SL1344ΔhilA:pMP220:napF	Prior, 2011
ΔSTM1808ΔtehB ΔyeaR ΔhmpA	SL1344ΔSTM1808::cat,ΔtehB ΔyeaR ΔhmpA::kan	Johnson

Table 2: Plasmids used

Plasmid	Genotype	Source
pcP20	Temperature-sensitive replication and thermal induction of FLP synthesis, Amp^R, Cat^R	Cherepanov & Wackernagel, 1995
pkD3	Amp^R, pANT-Sɣ derivative containing a FRT-flanked Cm^R	Datsenko & Wanner, 2000
pkD4	Amp^R, pANT-Sɣ derivative containing a FRT-flanked Kan^R	Datsenko & Wanner, 2000
pkD46	Amp^R, pINT-ts derivative containing araC-P_{araB} and ɣ, β, exo genes	Datsenko & Wanner, 2000
pMP220	Wide host-range promoterless-*lacZ* probe vector (Tet^R), low-copy number	Zaat *et al.*, 1987

2.3 Bacterial Culture Conditions

2.3.1 Media

All media was sterilized by autoclaving for a duration of 15 minutes at 121°C prior to use. Where necessary media was also supplemented with appropriate antibiotic(s), concentrations of which may be found in the ensuing section.

Luria Bertani (LB) Miller Broth (per L) (Bertani, 1951)

10g Tryptone

5.0g Yeast Extract

10.0g NaCl.

Solution was made up to 1L by addition of dH_2O and autoclaved.

Luria Bertani High Salt (LBS) Miller Broth (per L)

10g Tryptone

5.0g Yeast Extract

17.7g NaCl.

Solution was made up to 1L by addition of dH_2O and autoclaved.

Luria Bertani (LB) Agar (per L)

10g Tryptone

5.0g Yeast Extract

10.0g NaCl.

1.5% Agar

LB was prepared as described above. 200mL aliquots of this solution were divided into 500mL conical flasks containing 3g of 1.5% agar prior to autoclaving.

Lennox Broth (per L)

10g Tryptone

5.0g Yeast Extract

5g NaCl

Solution was made up to 1L by addition of dH_2O and autoclaved.

Green LB Agar (per L) (Maloy et al., 1996)

8g Tryptone

1g Yeast Extract

5g NaCL

Dissolved in 950mL dH_2O. 190mL aliquots of this solution were added to 500mL conical flasks containing 3g of 1.5% agar. Subsequent to autoclaving the following solutions were added:

21 mL (4mL per flask) 40% glucose (autoclaved)

25 mL (5mL per flask) 2.5% alizarin yellow G (autoclaved; heated until molten)

3.3 mL (0.65mL per flask) 2% aniline blue (filter sterilized)

M9 Minimal Media

M9 minimal media was prepared as follows.

33.91g Na_2HPO_4,

15g KH_2PO4

2.5g NaCl

5g NH_4Cl

Dissolved in 400mL dH_2O and autoclaved. This solution was then supplemented with the following:

30µg/1mL of Histidine

1µL/mL of 1M CaCl

20µl/mL $MgSO_4$.

2.5mL or 10mL of 1M $NaNO_3$

2.5mL or 10mL 1M Glycerol

2.3.2 Reagents

Z-Buffer

6.02mL $Na_2HPO_4.7H_2O$ (0.06M)

4mL $NaH_2PO_4.H_2O$ (0.04M)

1mL 1M KCl (0.01M)

0.1mL 1M $MgSO_4$ (0.001M)

0.175mL β-mercaptoethanol (BME) (0.05M)

Make up to 50mL with H_2O.

O-nitrophenol-β-D-galactopyranoside (ONPG)

4mg/ml ONPG was prepared by dissolving 0.012mg of O-nitrophenol-β-D-galactopyranoside in 3ml of phosphate buffer.

2.3.3 Antibiotic Stock

Antibiotic stocks were prepared to the following concentrations and stored in 1mL aliquots at 4°C. For tretracycline and chloramphenicol concentrations were achieved by dilution in ethanol instead of water.

Ampicillin	100μg mL^{-1}	Filter sterilized
Kanamycin	50μg mL^{-1}	Filter sterilized
Chloramphenicol	10μg mL^{-1}	
Streptomycin	100μg mL^{-1}	Filter Sterilized
Tetracycline	5μg mL^{-1}	

2.3.4 Overnight Culture

Using aseptic technique bacterial strains were streaked for single colonies on Luria-Bertani (LB) agar plates supplemented with appropriate antibiotic(s). Subsequent to streaking plates were incubated statically at 37°C overnight for

growth. Bacterial plates were stored at 4°C and used for a maximum of 10 days. Overnight cultures consisted of 10mL LB media supplemented with 10µL of appropriate antibiotic and inoculated with a single bacterial colony. These were incubated overnight at 37°C with shaking at 200 rpm.

2.3.5 Freezer stocks

Freezer stocks of bacterial strains were created and stored at -80°C using Microbank™ beads (ProLab Diagnostics) according to manufacturers instructions.

2.4 Polymerase Chain Reaction (PCR)

PCR was performed in a Thermocycler as per the program listed in Table 3. Elongation time was adjusted following the general rule of one minute per kilobase-pair (kb). Where necessary annealing temperature was optimised to 5°C below primer Tm. Conditions of PCR are described in Table 4.

Table 3: PCR Reaction Components

Reagent	Mutagenesis PCR [µL]	Verification PCR [µL]
Forward Primer (3'-5')	1	0.5
Reverse Primer (5'-3')	1	0.5
Template DNA	5	5
Sigma Water	189	6.5
BIOMIX (Bioline) 2X	25	23.5
Total volume	50	25

Table 4: PCR Programme Details

Programme Stage	Temperature [°C]	Time
1) Initial Denaturation	96°C	3 minutes
2) Denaturation	96°C	3 minutes
3) Annealing	55°C*	30 seconds
4) Elongation	72°C*	1.30 seconds
5) Repeat steps 2-4	29 X	
6 Final Elongation	72 °C	4 minutes

*Annealing temperature was altered depending on the Tm of primer sequences. *Elongation time was adjusted to at least 1-minute per kb of PCR product.

2.4.1 Colony PCR

To obtain chromosomal DNA a single bacterial colony was taken from a freshly streaked plate and resuspeded in 100µL of Sigma Water by vortexing. Subsequent solutions were boiled for 5 minutes at 100°C to lyse the cell before centrifugation for 2 minutes at 14600 rpm. 5µL of the resultant supernatant was used as template DNA.

2.4.2 PCR product purification (Miniprep)

PCR products were purified using a QIAquick PCR Purification Kit^{TM} (Qiagen) as per manufacturers instructions to eliminate excess PCR reaction reagents. DNA was bound to a centrifuge column by the mixture of PCR sample with binding buffer (PB) in a respective 1:5 volume. Following 1-minute centrifugation at 13,000 rpm and removal of the eluted supernatant, 0.75mL of PE buffer was added and the column centrifuged for a further minute before the supernatant was again discarded. Purified DNA was eluted by the addition of 50µL of Sigma Water to the column. Concentration of the purified product was determined by Nanodrop analysis and purified DNA stored at -20°C.

2.5 Plasmid purification (Miniprep)

Plasmid purification was achieved by use of a QIAprep Spin Miniprep Kit (Qiagen) according to manufactures instructions. Bacteria containing the desired plasmid were streaked for single colonies on an agar plate containing appropriate antibiotic. The following day 10mL LB supplemented with 10µl of appropriate antibiotic was inoculated with a single colony and grown overnight. 1mL of this culture was centrifuged at 14,000 rpm for 1 minute at

room temperature. The supernatant was discarded and the pellet resuspended in residual solution before a further 1ml of culture was added and the processes repeated. Once a sizeable pellet was achieved it was resuspended in 500µL of Buffer P1, mixed and 500µL of P2 buffer added. 700µL of buffer N3 was subsequently added and the solution centrifuged for 10 minutes at 13,000 rpm, after which the supernatant was applied to a centrifuge column. This column was then centrifuged and flow-through discarded before the column was washed and spun with 500ul PB buffer and 750µL PE buffer respectively. 50µL of Sigma Water was then applied to the column to elute the DNA.

2.6 Green Plates

Green plates containing a pH specific dye were used in the identification of true-lysogens and differentiation from pseudo-lysogens following phage transduction. Plates were prepared according to Maloy *et al*., 1996. Briefly, green agar was prepared using 8g Tryptone, 1g Yeast Extract, 5g NaCL with 1.5% (w/v) agar per 1 L dH_2O and autoclaved. 21mL of 40% (w/v) glucose (F/S), 25mL of 2.5% (w/v) alizarin yellow G and 3.3 mL of 2% (w/v) aniline blue (f/s) were added following autoclaving. Appropriate antibiotic was added if necessary. True-lysogens were identified on green plates by their light green colour.

2.7 Bacterial Growth Curves and Spectrophotometry

Bacterial growth curves were performed in 250mL conical flasks containing 50mL of LB broth. Flasks were inoculated with aliquots of LB overnight culture

normalized to an OD of 0.02 at 600nm and grown for 8 hours at 37°C, 200 rpm. 1mL of culture was taken from flasks at 1-hour intervals and transferred to a 1.5mL plastic cuvette by aseptic technique. The optical density of culture samples at 600nm (OD_{600}) was measured in a spectrophotometer (Molecular Devices, SpextraMax M5) using 1mL of sterile LB as a reference. Once cultures had reached an OD_{600} exceeding 1.0 1mL samples were diluted in a 1:10 ratio with sterile LB. Following 8 hours of growth, cultures were incubated overnight and a further measurement taken at 24 hours.

2.8 Agarose gel electrophoresis

Agarose gels were prepared for the separation and visualisation of PCR products as follows; 10x TBE buffer (106g Tris base, 55 g boric acid, 40mL EDTA, made up to 1L with dH_2O) was diluted 1:10 to form a 1 x TBE buffer solution.

The gel was prepared by adding 1% agarose (w/v) to 1x TBE solution in a conical flask which was then microwaved until agarose was fully dissolved.

Once cool, 0.04% (v/v) Ethidium Bromide was added to the agarose solution and mixed. The solution was then poured into a gel cast with comb and left to solidify at room temperature. The comb was then removed and the gel placed in an electrophoresis tank (Sub-cell GT, BIO-RAD)

2.9 Mutant Construction

2.9.1 Mutagenesis via Phage- λ Red Recombination

De novo mutagenesis was performed using the λ Red Recombination system to replace chromosomal genes of interest (GOI) in the *S.* Typhimurium strain

SL1344 with antibiotic resistant cassettes. Briefly, this technique utilizes three phage-λ Red proteins, γ (Gam), exo (Exo) and β (Beta) to recombine linear DNA containing short homologies with appropriate target sequences. γ prevents *E. coli* nucleases from degrading the linear double-stranded DNA (dsDNA) whilst Exo degrades this dsDNA to leave single-standed DNA that β may then bind to and facilitate recombination via annealing to the homologous genomic target site (Mosberg *et al*, 2010; Sawitzke *et al*, 2007).

Plasmid DNA encoding resistance cassettes for either chloramphenicol (pKD3) or kanamycin (pKD4) were amplified via PCR. Amplification was achieved using the PCR protocol described in Table 4, with primers listed in Table 5. Primers contained a 5' 40bp sequence homologous to regions upstream or downstream of the GOI linked to a 3' sequence homologous for the antibiotic resistance cassette to be amplified. This process is shown in Figure 11. The resultant linear PCR product with henceforth be referred to as the mutant construct and was PCR purified in preparation for electrophoresis.

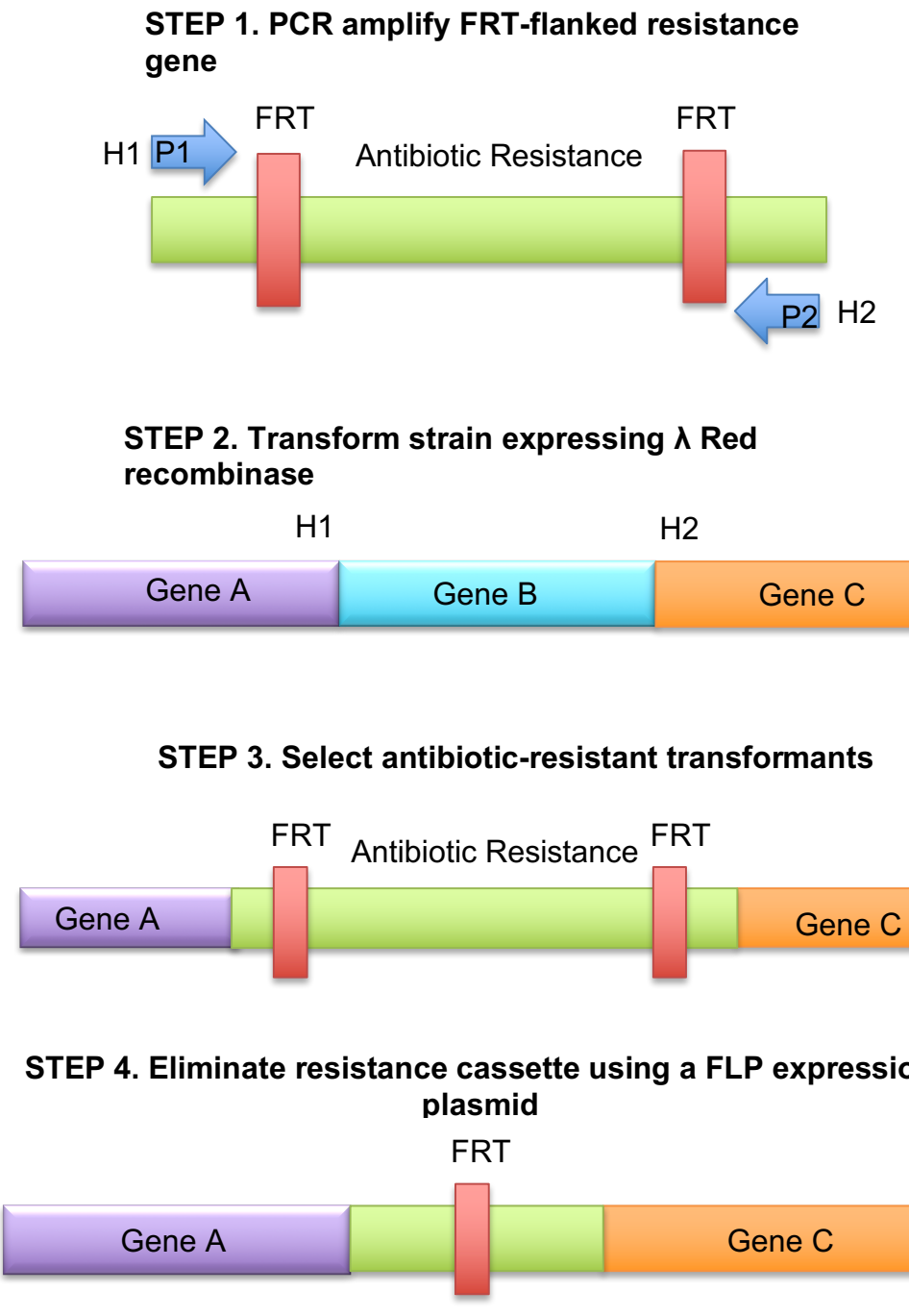


Figure 11: Mutant construction via Lambda Red Recombineering
H1 and H2 = Homology regions
P1 and P2 = Priming sites

1. Antibiotic cassettes amplified by PCR from pKD3 or pKD4
2. Resultant PCR products electroporated into the SL1344 containing pKD46.
3. Chloramphenicol and kanamycin resistant colonies identified via plating on antibiotic plates.
4. Mutants transduced into a clean *S.* Typhimurium SL1344 background to avoid further recombination events. Loss of the targeted gene was confirmed by PCR.

(Figure adapted from Datsenko & Wanner, 2000).

2.9.2 Electroporation

Electrocompetent cells were prepared of the WT strain SL1344 containing the temperature sensitive plasmid pKD46, which encodes the phage λ red recombination proteins. A single colony of SL1344 pkD46 was grown overnight at 30°C in 10mL LB broth containing 10µL ampicillin. A 1:100 dilution of this overnight was used to inoculate 50mL of Lennox Broth supplemented with 50µL Ampicillin and 50µL 1mM L-arabinose. This culture was then incubated at 30°C until it reached an OD_{600} of 0.6 at which point it was transferred into a falcon tube and centrifuged for 15 minutes at 4000 rpm, 4°C. The resultant pellet was re-suspended in 25µL of 10% (v/v) ice-cold glycerol before centrifugation and washing was repeated twice more. Cells were kept cold throughout this process to preserve their competency. Subsequent to the final wash, the pellet was resuspended in residual 10% glycerol. Solution containing the resuspended pellet was then divided into 100µL aliquots to which 10µL of PCR product was added. This mixture was then transferred to an electroporation cuvette and electroporation performed using a BIO-RAD MicroPulser at a voltage of 2.5kV. Immediately after electroporation, 1mL of sterile LB was added to the cuvette and the mixture pipetted into an Eppendorf tube. Electroporated cells were then incubated at 37°C for an hour to allow recombination. 100µL of cells was then spread on agar plates containing the appropriate antibiotic and incubated overnight at 37°C. Resultant colonies were patched onto a new antibiotic plate the following day and subsequently checked by Colony PCR, as described above, to determine whether mutagenesis had been successful.

2.9.3 P22 mediated transduction

Successfully mutated DNA was transduced into a clean WT SL1344 background using the P22 bacteriophage to prevent further recombination events from occurring. A single colony of the desired mutant, was used to inoculate 10mL of LB with appropriate antibiotic and this culture grown overnight. The following day 10µL of this overnight was subcultured into 10mL of sterile LB and grown for 1 hour at 37°C. 20µL of SL1344 P22 bacteriophage was then added and the culture grown for a further 6 hours to allow cell infection. Following this, 1mL of $CHCl_3$ was added to the culture and gently mixed before being left at 4°C for a minimum of 4 hours. Following incubation the solution was centrifuged for 15 minutes at 4°C and the resultant supernatant pipetted into a 15mL falcon tube to create a phage stock of the mutant. 10µL of this phage stock was then added to 100µL of SL1344 and incubated for 45 minutes at 37°C before streaking into an appropriate antibiotic plate. This plate was then incubated overnight at 37°C and resultant single colonies streaked onto a green antibiotic plate and again incubated. True-lysogens were identified the following day by their light-green colour and re-streaked onto a fresh green plate that was then incubated. True-lysogens were again identified and streaked onto an LB plate with appropriate antibiotic, following incubation and growth, single colonies were PCR checked by Colony PCR to ensure that they contained the desired mutation.

2.9.4 Double mutant construction

Double mutants were created from two single mutant strains with different antibiotic cassettes. 10mL LB containing the appropriate antibiotic was inoculated with a single colony of one of the two mutant strains. 10µL of this was then subsequently subcultured into fresh 10mL LB the following day and grown for an hour as described above. 10µL of this culture was then added to 20µL of phage stock of the second mutant and the protocol continued as described in 2.9.3.

Table 5: Primers used for deletion mutagenesis

Gene	Forward Primer	Reverse Primer
STM1586	TGTGAAGCAGCTATACACGTTTTTATCAAAGGGAGTCGTCGTGTAGGCTGGAGCTGCTTC	TAAAGAGATGACTGGAGGGGTTTCCCCCTCCATACCCTATCATATGAATATCCTCCTTAG
STM1250	ATAAGCCAGTTTTTTGTTTCAGCGGTGACTGAAACATATTGTGTAGGCTGGAGCTGCTTC	TAGTCACGAATTTAAGTGGTTAACGAAAGTCTCATCCCGTCATATGAATATCCTCCTTAG
STM2804	TAGTGCTACAGTGCTCGTCATCAACACGAGGAGAAACATG GTGTAGGCTGGAGCTGCTTC	ACAGAGTTGGCTGACATCAGCGCCATCCGGCAACAATGCGCATATGAATATCCTCCTTAG
STM4552	TATGATATTGGTTATCATTATCAATTCCAGAGGTGAAACCGTGTAGGCTGGAGCTGCTTC	CTCGCACCAATTATCTTATCCTTCCTTTGTCTCTTCATTTCATATGAATATCCTCCTTAG
IbpA-IbpB	ATGATGGAAGGTCTGACATTCTCGCTGATTTCAGGAGTTTGTGTAGGCTGGAGCTGCTTC	AAAGCCCCGCCATCTCTGGCGGGGCAAGGCAAGGAGCTCACATATGAATATCCTCCTTAG

STM1250-STM1251	ATAAGCCAGTTTTTTGT TTCAGCGGTGACTGAAA CATATTGTGTAGGCTGG AGCTGCTTC	GACAATGAAGGCCCGT CTAAACGGGCCTCCATT AACGCGACATATGAATA TCCTCCTTAG
ycfR	AATGCTTACGACACCCA TTCATCTGCTAAAGGTC ATCACTGTGTAGGCTGG AGCTGCTTC	TTATTGTGGCAATCGCA GCGGCATTAATGAGGG TTAATGCCATATGAATA TCCTCCTTAG
ybiJ	ACATCGTCCCATAAACA GAATAACCTGCGAGAG ATTAATCGTGTAGGCTG GAGCTGCTTC	AACAGCGATGTTACAGC CTGAAATCTGGCGGGC AGGTAAA CATATGAATATCCTCCT TAG
STM3362	TATCCATGAGTCCAGGT TCACTTTTGCAGGATAT ATCCTC GTGTAGGCTGGAGCTG CTTC	TACGCTATCCGGCTTAC AAGGCATCTGTAGGCT GGATAAA CATATGAATATCCTCCT TAG
NapF	GAATTCTCTTCATTTGAT TGT	CTGCAGGATATCGCCCT TCC

2.9.5 'Flipping out' of the antibiotic resistance cassette

To combine two or more deletion strains containing the same antibiotic resistant cassette it was necessary to first 'flip out' the cassette. Electrocompetent cells of one strain of interest were created as described in 2.9.2. 5µL of pCP20 was then added to 100µL aliquot of electro-competent cells and electroporated as previously described. Cells were then incubated statically at 30°C for three hours to recover and for expression of the temperature sensitive Pcp20 plasmid. This plasmid encodes the Flp recombinase enzyme of *Saccharomyces cerevisiae* which recognises the Flp recombinase targets (FRT) flanking the antibiotic resistance cassette. Production of the FLP recombinase enzyme results in excision of the antibiotic cassette (Ellermeier *et al*, 2002). Following incubation, 100µL of

cells were spread onto LB plates containing Ampicillin (Amp) and incubated overnight at 30°C. The next day colonies were grown statically in 10mL of LB at 45°C to eliminate the Pcp20 plasmid, then diluted 1/5 and 10 or 100µL of this dilution spread on LB plates which were incubated at 37°C overnight. Following this, colonies were patched onto LB, Amp and Cat or Km pates in immediate sucession and again incubated at 37°C overnight. Colonies which only gew on LB were patched onto a new LB plate and grown overnight at 37°C prior to PCR check with verification and internal cassette primers for absence of the cassette.

2.10 Sensitivity Assays

2.10.1 H_2O_2 Sensitivity Assay

A 96 well microplate was set up as follows. 100µL of LB media was added to the entire top row as a reference. 30% H_2O_2 was diluted in LB to the desired concentration and 100µL of this solution then added to each well in the first column of the plate. 50µL of LB was added to all remaining wells and a two-fold serial dilution performed by taking 50µL of the H_2O_2 solution from the first column and pipetting along the row, mixing thoroughly in between. 50µL of overnight culture normalized to an OD_{600} of 0.02 was then added to each well exempting the reference. The microplate was placed in a plate reader and shaken before an initial OD_{600} reading was taken. Following the initial reading the plate was incubated statically at 37°C and a reading taken each hour.

2.10.2 DetaNONOate Sensitivity Assay

Assays were performed as described above, using DetaNonoate in place of H_2O_2. An initial concentration of 50mM DetaNonoate was used and a two-fold serial dilution again performed. Assays were carried out aerobically and also anaerobically by adding 100µL aliquots of mineral oil to each of the wells.

2.10.3 Oxidative Stress Susceptibility Assay

Assay technique was adapted from the Oxidative stress susceptibility assays in Testerman *et al,* 2002. S. Typhimurium strains were grown overnight at 37°C and 200 rpm shaking before being diluted to a density of 5 x 10^5 cfu ml^{-1} in sterile PBS. Diluted strains were then challenged with either 0mM or 4mM H_2O_2 and left to stand at room temperature. 100µL aliquots were removed at 30-minute intervals for the first three hours and then every hour for 7 hours and then again at 24 hours. Aliquots were diluted in 10mL PBS before being plated as 10µL spots on LB agar plates. Plates were then grown at 37°C overnight before counting to quantify colony forming units (cfu).

2.10.4 Nitrosative Stress Susceptibility Assay

S. Typhimurium strains were grown as previously described in 2.10.3 and again diluted to a density of 5 x 10^5 cfu mL^{-1} in sterile PBS. Diluted strains were then challenged with either 0mM or 7mM detaNONOate and incubated statically at 37°C. The assay was then performed as previously described excepting that samples were taken every hour for the first 7 hours, then at 30-minute intervals for 3 hours and then again at 24 hours.

2.11 β-galactosidase Assay

2.11.1 LB and LBS Media

β-Galactosidase Assays were carried out using strains of *S.* Typhimurium transformed with the pMP220 reporter plasmid encoding the *nap-lacZ* fusion. Universals containing 10mL of either LB or LBS media were inoculated with a single colony, and 10μL of tetracycline. Cultures were then grown micro-oxically and statically at 37°C overnight. 2mL aliquots of the cultures were added to micro-centrifuge tubes, centrifuged at 6000rpm for 10 minutes and pellets resuspended in 2mL chilled Z-buffer. 1mL of the diluted cells were then added to cuvettes and the OD read at 600nm. 100μL chloroform and 50μL of 0.1% sodium dodecyl sulphate (SDS) was added to the remaining 1mL of diluted culture and this mixture vortexed before heated to an equilibrium at 28°C for 5 minutes. Samples were then removed from the heat block and the β-galactosidase activity reaction started by the addition of 0.2mL ONPG solution. The samples were then vortexed and placed back in the heat block. Once a yellow colour similar to that of LB had developed the reaction was stopped by the addition of 0.5mL 1M Na_2CO_3. This solution raises the pH to 11 and therefore abrogates the enzymatic reaction. The time taken for the yellow colour change from the addition of the ONPG to the termination of the reaction with Na_2CO_3 was recorded. The 1mL of reaction mixture was then centrifuged for 5 minutes at 13000rpm to remove the chloroform and SDS before the OD was recorded at 550nm and 420nm. The reaction time, reaction volume and OD_{600}, OD_{420} and OD_{550} readings were then used to calculate the enzyme activity in Miller Units using the following formula:

$1000 \times (OD_{420} - (1.75 \times OD_{550})) / (\text{time (minutes)} \times \text{volume (mls)} \times OD_{600})$

2.11.2 M9 Minimal Media

β-galactosidase assays were performed in M9 minimal media containing either 10mL 1M Glycerol and 2.5mL 1M Nitrate or vice versa. 50mL of M9 minimal media was added to 50mL falcon tubes and inoculated with a single colony and 50μL of tetracycline. The culture was then sealed with parafilm and left to grow anaerobically and statically at 37°C for 20 hours. The entire 50mL culture was then spun down at 4000rpm for 20 minutes and the pellet resuspended in 2mL Z buffer. 1mL of this was added to a cuvette and the OD_{600} recorded. 100μL of the diluted cells was further diluted in 900μL of Z-buffer in a microcentrifuge tube before the addition of 100μl chloroform and 50μl SDS. The β-galactosidase assay was then carried out as described above.

CHAPTER 3: Construction of mutants and analysis of mutant sensitivity to oxidative and nitrosative stress

3.1 Introduction

It is apparent that the nitrosative stress response of *Salmonella* is critical to the bacterium's survival and pathogenicity within host organisms. Therefore, the mechanisms *Salmonella* employs to eliminate exogenous NO are a significant yet largely unknown area of study. Whilst the three metalloenzymes NrfA, NorV and HmpA remain the principle and most well characterised systems of NO detoxification in *Salmonella*, additional proteins are thought to play important functional roles in nitrosative stress resistance. NsrR-regulated genes are likely candidates due to their response to very low concentrations of NO (Karlinsey *et al*, 2012). As previously described, the NsrR transcriptional repressor contains a helix-turn-helix DNA binding domain, the binding of which is inhibited when the Fe-S clusters within NsrR are nitrosylated. Abrogation of NsrR binding occurs at very low NO concentrations, resulting in the transcription of genes such as *hmpA* (Gilberthorpe *et al*, 2007). Fairly recently two genes, *STM1808* and *ygbA*, both of which are encoded within the NsrR regulon, have been identified as necessary for nitrosative stress resistance in *S.* Typhimurium. Insertion mutations were created in *STM1808* and *ygbA* and the resulting mutant strains showed impaired growth in the presence of NO (Karlinsey *et al*, 2012). Such results suggest the involvement of other, yet unidentified, genes in NO detoxification besides hmpA.

ROS produced by macrophages are also capable of inhibiting or killing pathogens and *Salmonella* has means by which it disrupts iNOS activities, detoxifies ROS and repairs ROS damage (Burton *et al*, 2014). While the importance of directly bactericidal ROS in *Salmonella* killing remains vague,

oxidative stress is known to be important in the control of *Salmonella* infection (Vazquez-Torres *et al*., 2000). For instance, Hydrogen peroxide (H_2O_2) is able to move across bacterial membranes and cause oxidative damage to bacterial components (Hébrard *et al*, 2009). While various oxidative stress defence mechanisms have been described in *Salmonella*, it remains a complex area in which much is still unknown.

The genes investigated within this study are proposed to be involved in some capacity in the nitrosative or general stress response of *Salmonella.* They were initially identified in a microarray previously performed by Rowley's group, which revealed that *ybiJ, ycfR, STM1250, STM4552, ibpA* and *STM1586* were induced in *S*. Typhimurium under anaerobic conditions upon the addition 40μM aqueous NO (Figure 12). This induction of expression implies some contribution to nitrosative stress resistance, or the general stress response of *S.* Typhimurium. It is possible that some of the genes identified in the nitrosative stress assay might also function as oxidative stress resistant genes.

This project therefore aimed to characterise deletion mutants of the aforementioned genes and others previously implicated in NO detoxification and sought to examine their sensitivity to oxidative and nitrosative stress.

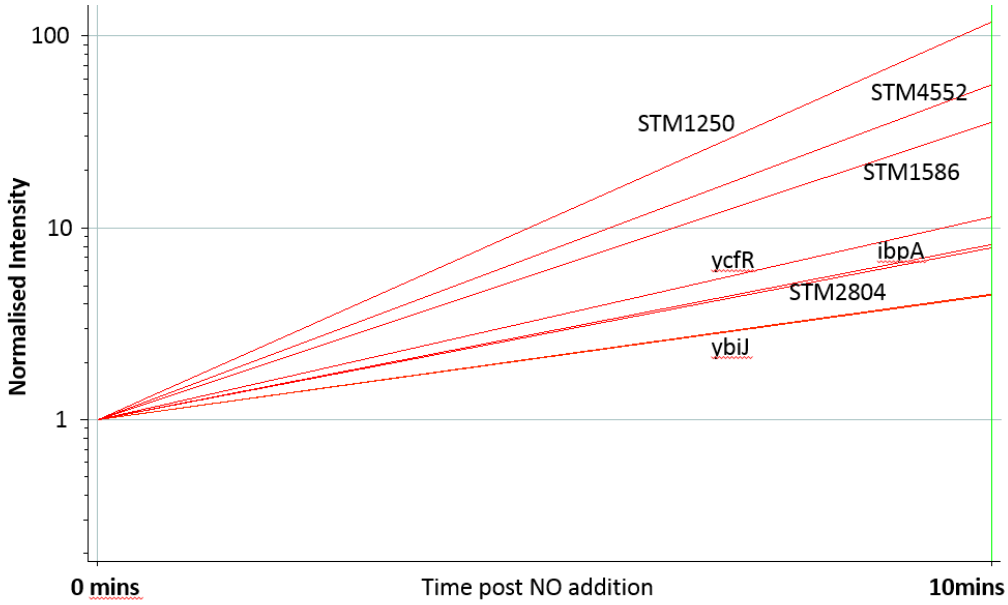


Figure 12: *Salmonella* genes up-regulated under nitrosative stress. Preliminary data from a microarray showed that upon the addition of 40μM aqueous NO, the *S.* Typhimurium genes *STM1250, STM4552, STM1586, STM2804, ycfR, ybiJ and ibpA* were up-regulated.

STM1586, STM3362* and *STM4452.

Little is known about the majority of genes identified in the before mentioned microarray; for instance, *STM1586* and *STM3362*, which are putative periplasmic proteins, and *STM4452*, which is a putative inner membrane protein. None of these three genes are thought to be part of the NsrR regulon however both *STM1586* and *STM4452* were upregulated in response to nitrosative stress. Whilst *STM3362* was not shown to be induced in the microarray, it contains a domain of unknown function, DUF1471, which bore

further investigation. The relevance of this DUF is described later within this chapter. Moreover, *STM3362,* known as *ychH* in *E. coli*, has been shown to be upregulated under chlorine oxidation in *S.* Typhimurium (Wang *et al*, 2010). A study in metabolically engineered *E. coli* also concluded that YchH (STM3362) was a stress protein, as it displayed significant sensitivity to H_2O_2, cadmium and acid, as well as involvement in biofilm formation (Lee *et al*, 2010).

YcfR

More is known about the outer-membrane protein YcfR. Studies in *E. coli* showed that *ycfR* expression was induced under several stress conditions, including oxidative stress. Further investigation showed that deletions of *ycfR* in *E. coli* resulted in strains with increased sensitivity to oxidative stress, as well as acid and heat, leading to the conclusion that *ycfR* is a multiple stress resistance protein (Zang *et al*, 2007). Wang *et al* (2009) further showed that *ycfR* was up-regulated in *E. coli* under oxidative stress from chlorine and H_2O_2. Studies conducted in *S.* Typhimurium showed that *ycfR* was induced by chlorine stress and that its deletion resulted in significantly reduced chlorine resistance (Salazar *et al,* 2013). As YcfR functions as a multiple stress resistance protein in E. *coli*, it may indeed perform a similar role in *Salmonella* (Deng *et al*, 2011). Evidence also suggests this protein may also be involved in biofilm formation, where it shows signification upregulation in biofilms (Deng *et al*, 2011; Zang *et al*, 2007).

YbiJ

YbiJ, a putative periplasmic protein, was also shown to influence *E. coli* biofilms and a deletion of *ybiJ* significantly reduced biofilm formation by *E. coli* (Hancock *et al*, 2010). Beyond this, little is known about its role in *Salmonella* or *E. coli* stress responses. It is notable however that YcfR, YbiJ and STM3362 all possess the same domain of uncharacterized function, DUF1471. Proteins containing this domain have been shown to play roles in bacterial stress response, biofilm formation and pathogenesis (Eletsky *et al*, 2014). Several members of this family, including *ycfR*, are up-regulated in *Salmonella* subsequent to the application of oxidative and other stresses (Eletsky *et al*, 2014). It has been proposed that DUF1471 proteins may have a direct or indirect influence on the characteristics of the cell surface; though more about their role and function in relation to stress remain unclear (Eletsky *et al*, 2014).

IbpA, IbpB, STM1250* and *STM1251

Significantly more research has been performed on *ibpA* and the closely related *ibpB*, two small heat shock chaperones which bind to denatured proteins, preventing further protein aggregation and leading to their reformation (Matuszewska *et al*, 2008). Unsurprisingly both *ibpA* and *ibpB* are up-regulated in *Salmonella* and *E. coli* in response to heat stress. These two proteins have a multiprotein structure which dissociates at high temperatures and aid in the refolding or degradation of damaged proteins (Lethanh *et al*, 2005).

Strains of *E. coli* which overexpressed *ibpA* and *ibpB* were found to be more resistant to both heat and superoxide stress, though not to hydrogen peroxide. However, further testing with *ibpAB*-disrupted strains showed them to be no more sensitive to these stresses than WT controls (Kitagawa *et al*, 2000). *Salmonella*, unlike *E*. coli, also produces two *ibpA* and *ibpB* homologues, *STM1250* and *STM1251* respectively. IbpA shares 35% sequence identity with STM1250 and IbpB shares a 31% sequence identity with STM1251. In order to observe a mutant phenotype all four genes must therefore be removed to ensure that these homologues do not compensate for each other and mask the effect of the deletion.

3.2 Aims

The first aim of this study was the construction of deletion strains for all genes of interest (GOI) detailed above. Once strains had been confirmed to have the desired mutations it was then necessary to ensure that they displayed no significant general fitness defects compared to the WT strain that would impact future results. The final aim of this chapter was to determine whether mutants demonstrated increased sensitivity to nitrosative and / or oxidative stress in comparison to the WT strain, therefore indicating involvement of the GOI in the response of *Salmonella* to these stressors.

3.3 Methods

3.3.1 Construction of gene deletions

Single deletion mutants of all the GOI were constructed as well as double mutant strains of *ibpA-ibpB* and *STM1250-STM1251* respectively. To try and

unpick functional redundancy, a quadruple mutant was then constructed of *ibpA, ibpB, STM1250* and *STM1251*, as well as a triple mutant of ST*M3362, ybiJ, ycfR* to examine the involvement of DUF471-containing proteins to stress responses in *S.* Typhimurium. Mutants were constructed using λ Red Recombination, a technique that utilizes the bacteriophage λ homologous recombination proteins to recombine linear DNA containing short homologies with appropriate target sequences. It is highly efficient in that it requires only 50 bases of homology for efficient recombination and can be used to make precise insertions, point mutations and deletions in *Salmonella* (Mosberg *et al*, 2010). The *Salmonella* strain used in this study is *Salmonella enterica* enterica serovar Typhimurium str. SL1344 and all subsequent mutants derived from this original WT strain. Targeted genes were excised and replaced with antibiotic resistance cassettes encoding resistance for either chloramphenicol (pKD3) or kanamycin (pKD4) and flanked with a flippase recognition target (FRT) to allow for later removal. These cassettes were first PCR amplified from the plasmids pKD3 and pKD4 using primers designed for the deletion of targeted genes. These primers may be found in Table 5. This resulted in a linear PCR product consisting of the antibiotic cassette and 40bp overhanging ends homologous to the start and end of the gene to be removed (Figure 11). This PCR product was then purified and electroporated into a *S.* Typhimurium SL1344 strain containing the pkD46 plasmid, which encodes the arabinose inducible recombinase enzymes of phage λ (γ, β, exo).

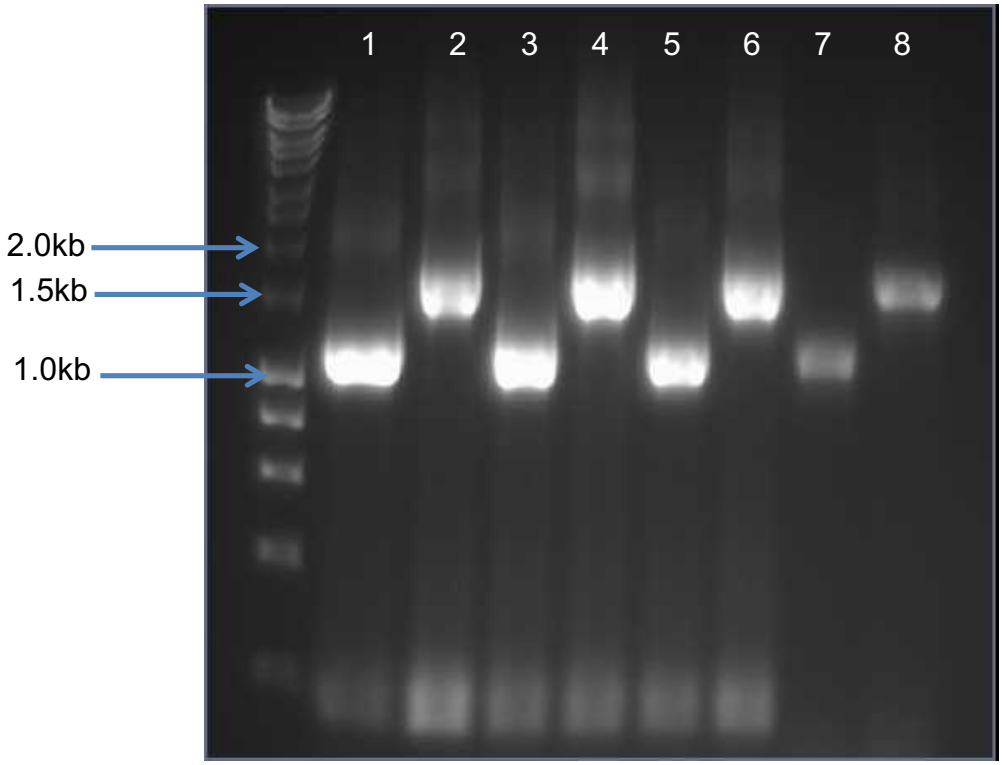


Figure 13: Creation of linear PCR product. Gel electrophoresis of mutagenesis primers and either pKD3 or pKD4 DNA.
Lane 1: pDK3 STM4552 Lane 2: pKD4 STM4552 Lane 3: pKD3 STM1250, Lane 4: pDK4 STM1250 Lane 5: pKD3 STM2804 Lane 6: pKD4 STM2804 Lane: 7: pDK3 STM1586 Lane: 8 pKD4 STM1586.

3.3.2 Confirmation of single gene knockouts

Mutants were confirmed by colony PCR using verification primers external to the loci where the mutation should have occured and internal primers which amplified the pKD3 or pkD4 casette (Figure 13). Once mutagenesis was confirmed, successful recombinants were transduced into a clean *S.* Typhimurium SL1344 background to prevent further recombination from occuring. These recombinants were then plated on green plates containing the dyes alizarin yellow G and aniline blue. Lysogenic colonies appear dark green on such plates, whilst true lysogens appear light green, allowing for their differentiation and selection. Mutant confirmation with external and internal primers was again performed following true-lysosgen selection (Figure 14). Mutant strains of *ycfR, ybiJ* and *STM3362* previously created were also re-confirmed by colony PCR (Figure 15).

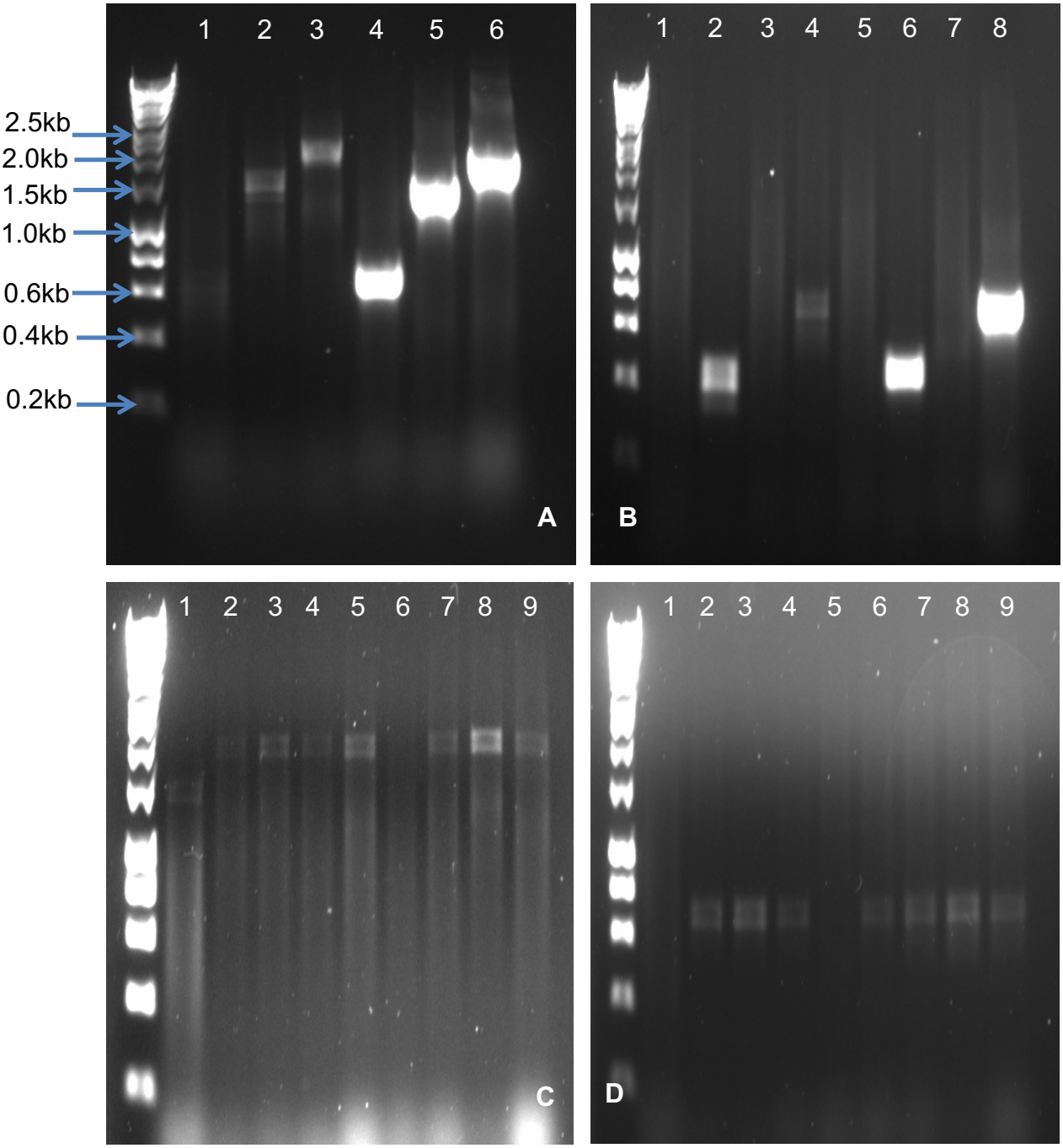


Figure 14 (A-D): Confirmation of novel single mutant strains. (A) PCR Check with external verification primers for mutant STM4552 and STM1250. Lane 1: WT STM4552, Lane 2: ΔSTM4552::cat, Lane 3: ΔSTM4552::kan, Lane 4: WT STM1250, Lane 5: ΔSTM1250::cat, Lane 6: ΔSTM1250::kan. (B) PCR check with internal verification primers for mutant STM4552 and STM1250. Lane 1: WT STM4552, Lane 2: ΔSTM4552::cat, Lane 3: WT STM4552, Lane 4: ΔSTM4552::kan, Lane 5: WT STM1250, Lane 6: ΔSTM1250::cat, Lane 7: WT STM1250, Lane 8: ΔSTM1250::kan. (C) PCR check with external verification primers for mutant STM1586 from numerous colonies. Lane 1: WT STM1586, Lane 2-9: ΔSTM1586::kan (D) PCR check with internal primers for mutant STM1586 from numerous colonies. Lane 1: WT STM1586, Lane 2-9: ΔSTM1586::kan.

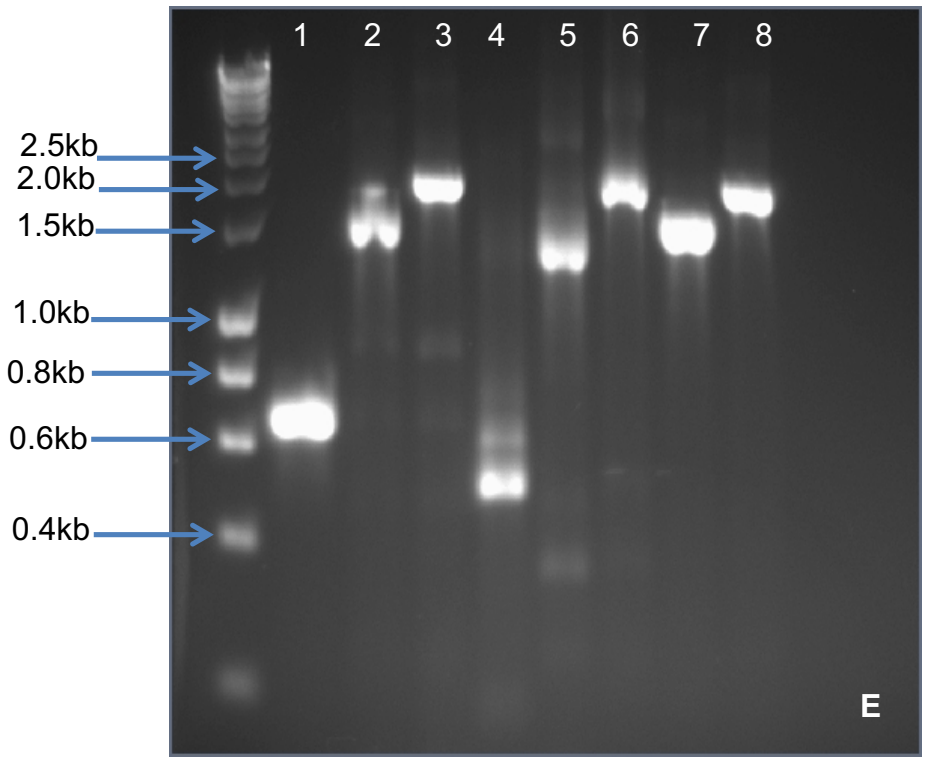


Figure 14 (E): Confirmation of novel single deletion strains (E) PCR confirming all single mutants with external verification primers. Lane 1: WT STM1250, Lane 2: ΔSTM1250::cat, Lane 3: ΔSTM1250::kan, Lane 4: WT STM4552, Lane 5: ΔSTM4552::cat, Lane 6: ΔSTM4552::kan, Lane 7: WT STM1586, Lane 8: STM1586::kan.

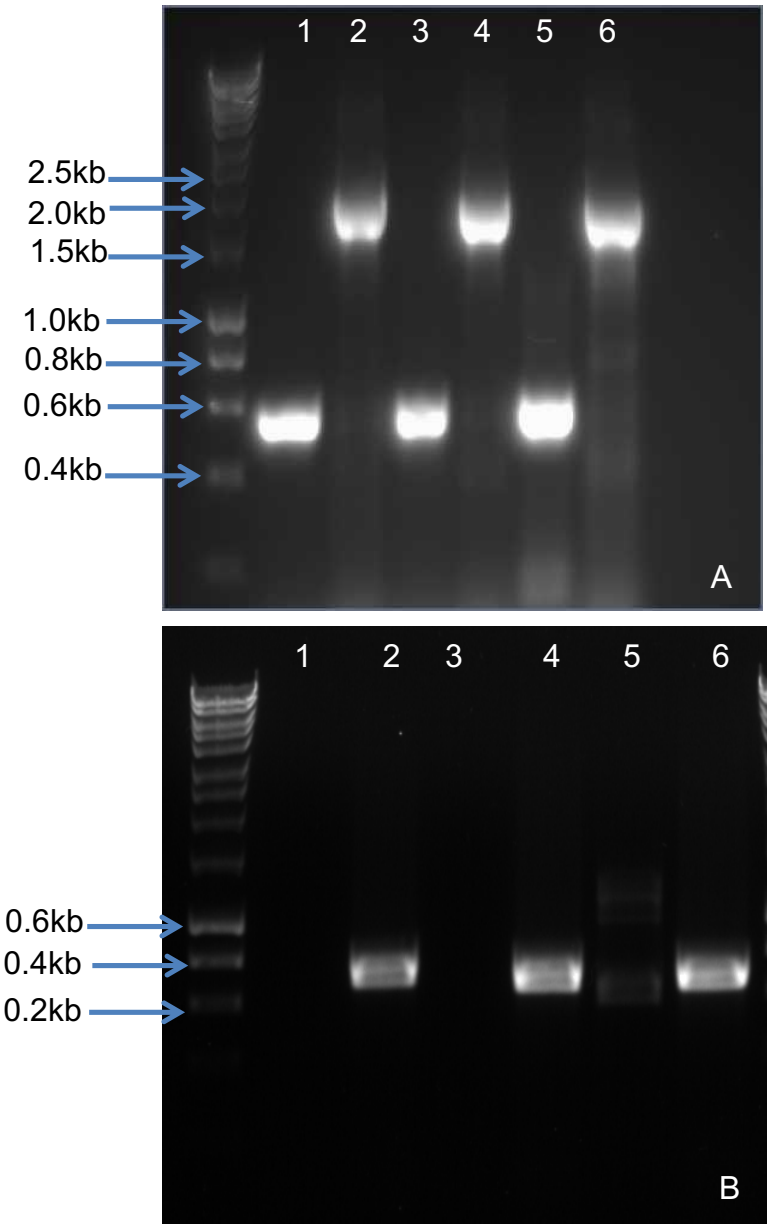


Figure 15: Confirmation of existing ΔybiJ, ΔycfR and ΔSTM3362. (A) PCR confirmation with external primers for the mutants of ybiJ, ycfR and STM3362. Lane 1: WT ybiJ, Lane 2: ΔybiJ::kan, Lane 3: WT ycfR, Lane 4: ΔycfR::kan, Lane 5: WT STM3362, Lane 6: ΔSTM3362::kan (B) PCR confirmation with internal primers for the mutants of ybiJ, ycfR and STM3362. Lane 1: WT ybiJ, Lane 2: ΔybiJ::kan, Lane 3: WT ycfR, Lane 4: ΔycfR::kan, Lane 5: WT STM3362, Lane 6: ΔSTM3362::kan.

3.3.3 Creation and confirmation of double, quadruple and triple mutants

Due to the adjacent location of the *ibpA* and *ibpB* and the *STM1250* and *STM1251* genes they could each be replaced simultaneously by one antibiotic resistance casette. The resultant recombinant was therefore a double mutant, either for Δ*ibpA-ibpB* or Δ*STM1250-STM1251*, elsewise construction was identical to single mutant creation.

From these double mutants it was possible to create a *ΔibpA-ibpB-STM1250-STM125*1 quadruple mutant. It was necessary to use mutants containing two different antibtiotic casettes and therefore *STM1250-STM1251* with resistance to chloramphenicol and *ibpA-ibpB* with resistance to kanamycin were used (Figure 16, Figure 17). The phage transduction step was carried out once more, using one double mutant as the donor strain and the other in place of the clean SL1344 background. The successful quadruple mutant was then confirmed by PCR using both external and internal verification primers (Figure 18).

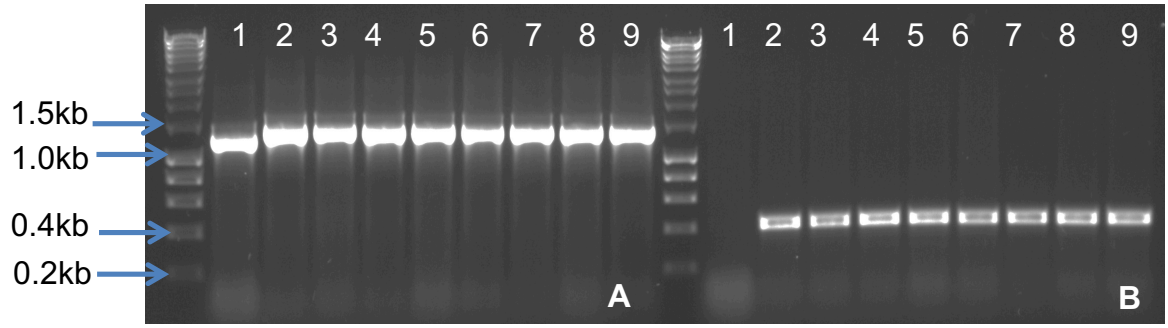


Figure 16: Confirmation of ΔSTM1250-STM1251::cat double mutant. (A) PCR check with external verification primers of numerous colonies for ΔSTM1250-STM1251::cat. Lane 1: WT STM1250-STM1251, Lane 2-9: ΔSTM1250-STM1251::cat. (B) PCR check wit internal verification primers of numerous colonies for ΔSTM1250-STM1251::cat. Lane 1: WT STM1250-STM1251, Lane 2-9: ΔSTM1250-STM1251::cat

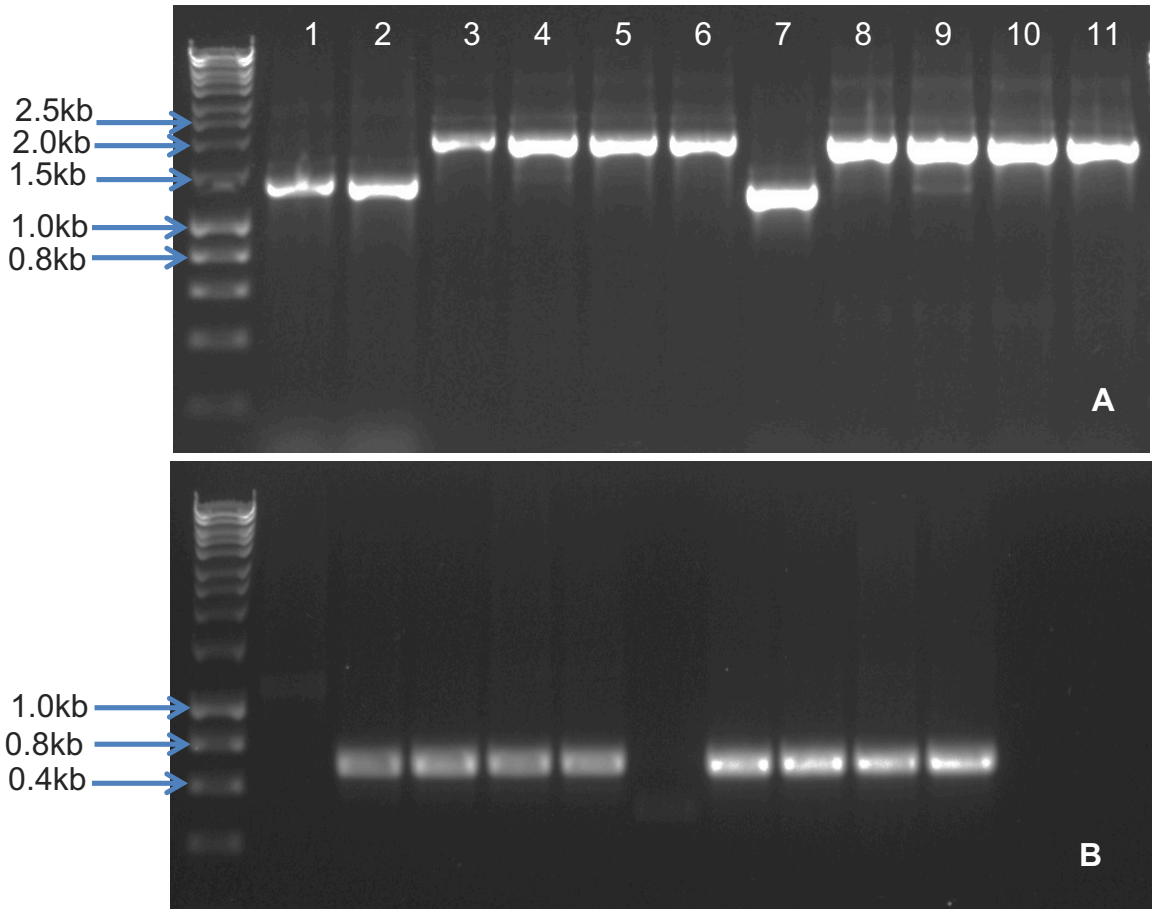


Figure 17: Confirmation of ΔIbpA-IbpB::kan and ΔSTM1250-STM1251::kan (A) PCR check with external verification primers for IbpA-IbpB and STM1250-STM1251. Lane 1: WT IbpA-IbpB, Lane 2: WT IbpA-IbpB, Lane 3-6: ΔIbpA-IbpB::kan, Lane 7: WT STM1250-STM1251, Lane 8-11: ΔSTM1250-STM1251::kan (B) PCR check with internal verification primers for IbpA-IbpB and STM1250-STM1251. Lane 1: WT IbpA-IbpB, Lane 2-5: ΔIbpA-IbpB::kan Lane 6: WT STM1250-STM1251, Lane 7-10: ΔSTM1250-STM1251::kan.

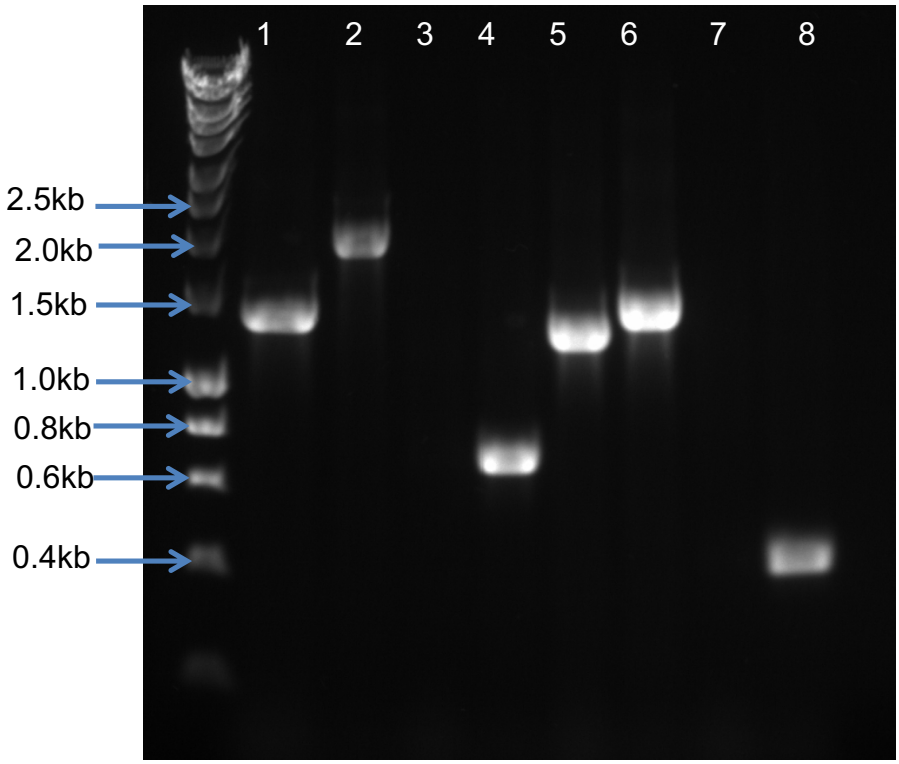


Figure 18: Confirmation of ΔIbpA-IbpB - STM1250-STM1251 quadruple mutant with both internal and external verification primers. Lane 1: WT IbpA-IbpB (external), Lane 2: ΔIbpA-IbpB::kan (external), Lane 3: WT IbpA-IbpB (internal), Lane 4: ΔIbpA-IbpB::kan (internal), Lane 5: WT STM1250-STM1251 (external), Lane 6: ΔSTM1250-STM1251::cat (external), Lane 7: WT STM1250-STM1251 (internal), Lane 8: ΔSTM1250-STM1251::kan

The *STM3362-ycfR-ybiJ* triple mutant construction required a further step in the process, where the antibiotic casette in Δ*STM3362* was first 'flipped out' (Figure 19). This was accomplished by electroporating the plasmid pcP20 into competent cells of Δ*STM3662*. This plasmid encodes the temperature sensitive yeast Flp recombinase gene, *flp,* and resistance to ampicillin. Following induction at the correct temperature the flp protein recognizes the FRT sequences flanking the antibiotic casette and flips this casette out. The resulting clean mutant of Δ*STM3362* was phage transduced with lysate raised on Δ*ycfR::cat* (Figure 20), creating a Δ*STM3362-ycfR* double mutant which was confirmed by colony PCR (data not shown). This double mutant was then tranduced with Δ*ybiJ*::kan to create *STM3362-ycfR-ybiJ*. The successful triple mutant was confirmed with both internal and external primers (Figure 21).

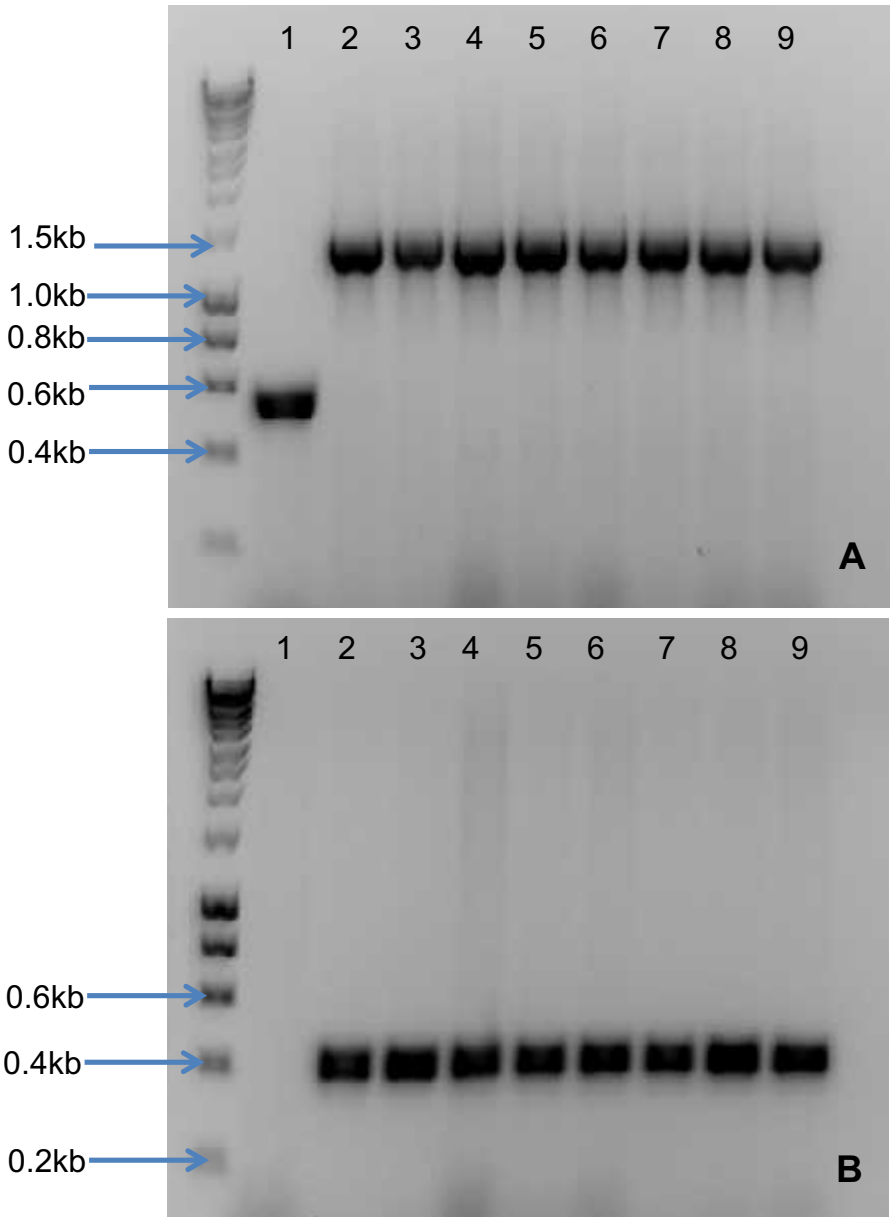


Figure 19: PCR check confirming ΔycfR::cat (A) PCR check with external primers for ΔycfR::cat, Lane 1: WT, Lane 2 – 9: ΔycfR::cat (B) PCR check with internal primers for ΔycfR::cat, Lane 1: WT ycfR, Lane 2-9: ΔycfR::cat

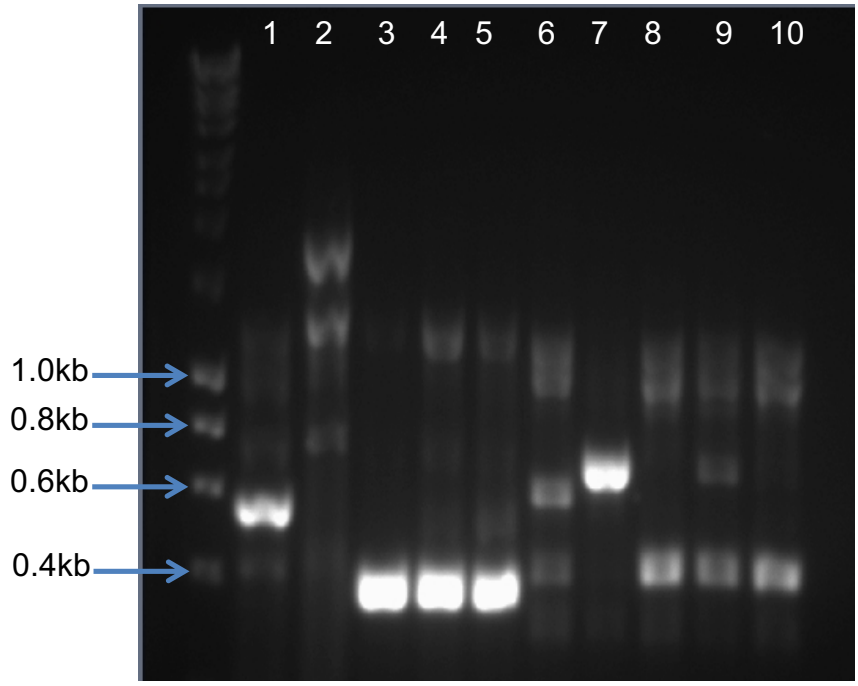


Figure 20: PCR check with internal and external verification primers for flipped out ΔSTM3362::kan Lane 1: WT STM3362 (external), Lane 2: ΔSTM3362::kan (external), Lane 3-5: flipped STM3362 (external), Lane 6: WT STM3362 (internal), Lane 7: ΔSTM3362::kan (internal), Lane 8-10: flipped STM3362 (internal).

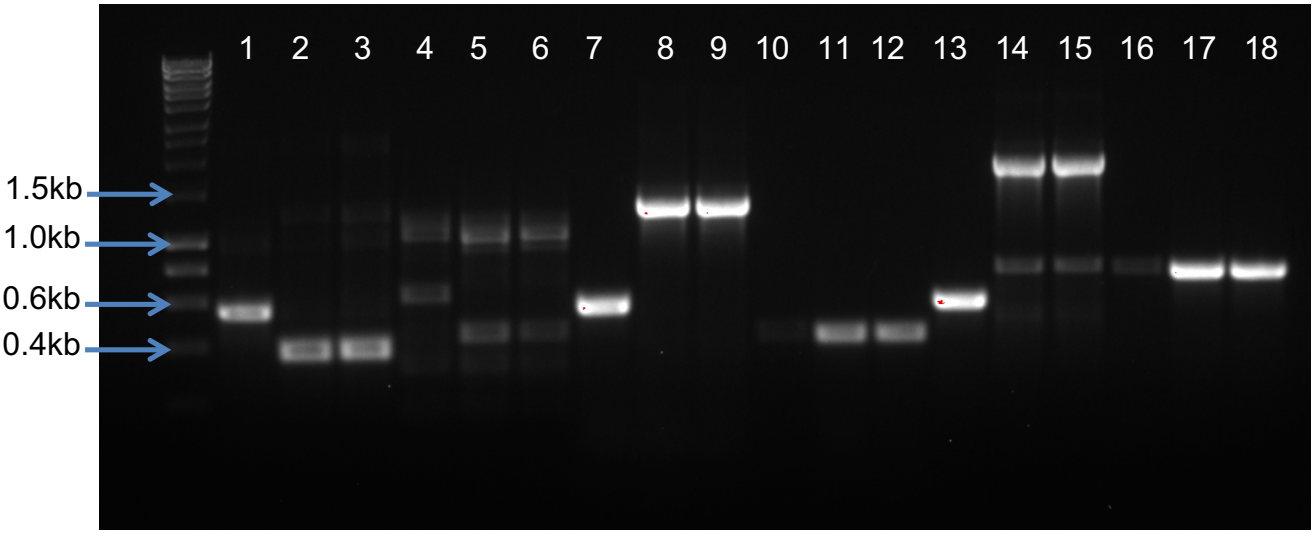


Figure 21: PCR check with internal and external verification primers for ΔSTM3362-ycfR-ybiJ triple mutant. Lane 1: WT STM3362 (external), Lane 2: STM3362 flipped (external), Lane 3: STM3362 flipped (external), Lane 4: WT STM3362 (internal), Lane 5: STM3362 flipped (internal), Lane 6: STM3362 flipped (internal), Lane 7: WT ycfR (external), Lane 8: ΔycfR::cat (external), Lane 9: ΔycfR::cat (external), Lane 10: WT ycfR (internal), Lane 11: ΔycfR::cat (internal), Lane 12: ΔycfR::cat (internal), Lane 13: WT ybiJ (external), Lane 14: ΔybiJ::kan (external), Lane 15: ΔybiJ::kan (external), Lane: 16 WT ybiJ (internal), Lane 17: ΔybiJ::cat (internal), Lane 18: ΔybiJ::cat (internal).

3.3.4 Aerobic Growth Curves in nutrient sufficient LB media

Growth curves were carried out in nutrient sufficient LB media under aerobic conditons for all mutant strains alongside the SL1344 *Salmonella* WT, facillitating growth comparison. Strains were normalised to an OD of 0.02 at 600nm in 50mL of LB broth and grown for 8 hours at 37°C, 200 rpm with OD_{600} measured at 1-hour intervals to determine bacterial growth.

3.3.5 Nitrosative and Oxidative Stress Sensitivity Assays

The effect of oxidative and nitrosative stress on deletion mutants in comparison with the *S*. Typhimurium WT strain SL1344 was determined through various sensitivity assays. Hydrogen peroxide was used as a source of oxidative stress whilst nitrosative stress was induced by use of Deta-NONOate. NONOates are compounds containing three sequential nitrogen atoms, enabling them to release NO when in solution. The majority of NONOates are stable in alkaline solution above pH 8.0 and generate NO when the pH is lowered. Per 1M of parent compound, Deta-NONOate releases 2M of NO and has a half-life of 20 hours at 37°C or 56 hours at room temperature when at pH 7.4.

3.3.5.1 Hydrogen Peroxide Sensitivity Assays

H_2O_2 sensitivity assays were performed using serial dilutions of 30% H_2O_2 in 96 well plates. Cultures of mutant strains Δ*STM1250,* Δ*STM1250-STM1251,* Δ*ibpA-ibpB* and Δ*ibpA-ibpB-STM1250-STM1251* and the WT strains were normalized to a starting density of 0.02 OD_{600} before addition to the serial

dilution. The microplate was incubated statically at 37°C and the OD_{600} of cultures taken via plate reader each hour.

3.3.5.2 DetaNonoate Sensitivity Assays

DetaNONOate sensitivity assays were performed much the same way as described above, excepting that DetaNONOate was utilised as a source of stress. An initial concentration of 50mM DetaNonoate was used and a two-fold serial dilution again performed. The assay was carried out under both aerobic and anaerobic conditions; the later of which was accomplished by the addition of 100µL of mineral oil to each of the wells.

3.3.5.3 Oxidative Stress Susceptibility Assays

Oxidative stress susceptibility assays were adapted from Testerman *et al*, 2002. Mutant and WT strains were challenged with either 0mM or 4mM H_2O_2 and left to stand at room temperature. Aliquotes were removed at 30 minute intervals for the first three hours and diluted before being plated as 10µL spots on LB agar plates. This then continued at 1 hour-intervals for 24 hours. Susceptibility of strains to oxidative stress was determined by cfu on LB agar plates after incubation at 37°C overnight.

3.3.5.4 Nitrosative Stress Susceptibility Assays

Stress susceptibility assays were again adapted from Testerman *et al*, 2002. The assay was performed as previously described for oxidative stress excepting that nitrosative stress was investigated instead by use of the NO donator detaNONOate. Strains were exposed to either 0mM or 7.5mM

detaNONOate. Aliquots were taken every hour for the first 7 hours, then at 30-minute intervals for 3 hours and then again at 24 hours.

3.4 Results

3.4.1 Mutant growth is comparable to WT in LB nutrient rich media

Wild-type and mutant strains were grown in nutrient rich LB media to ensure there were no major issues with general fitness of the strains. All mutants showed very similar growth to wild-type under these conditons (Figures 22, 23, 24).

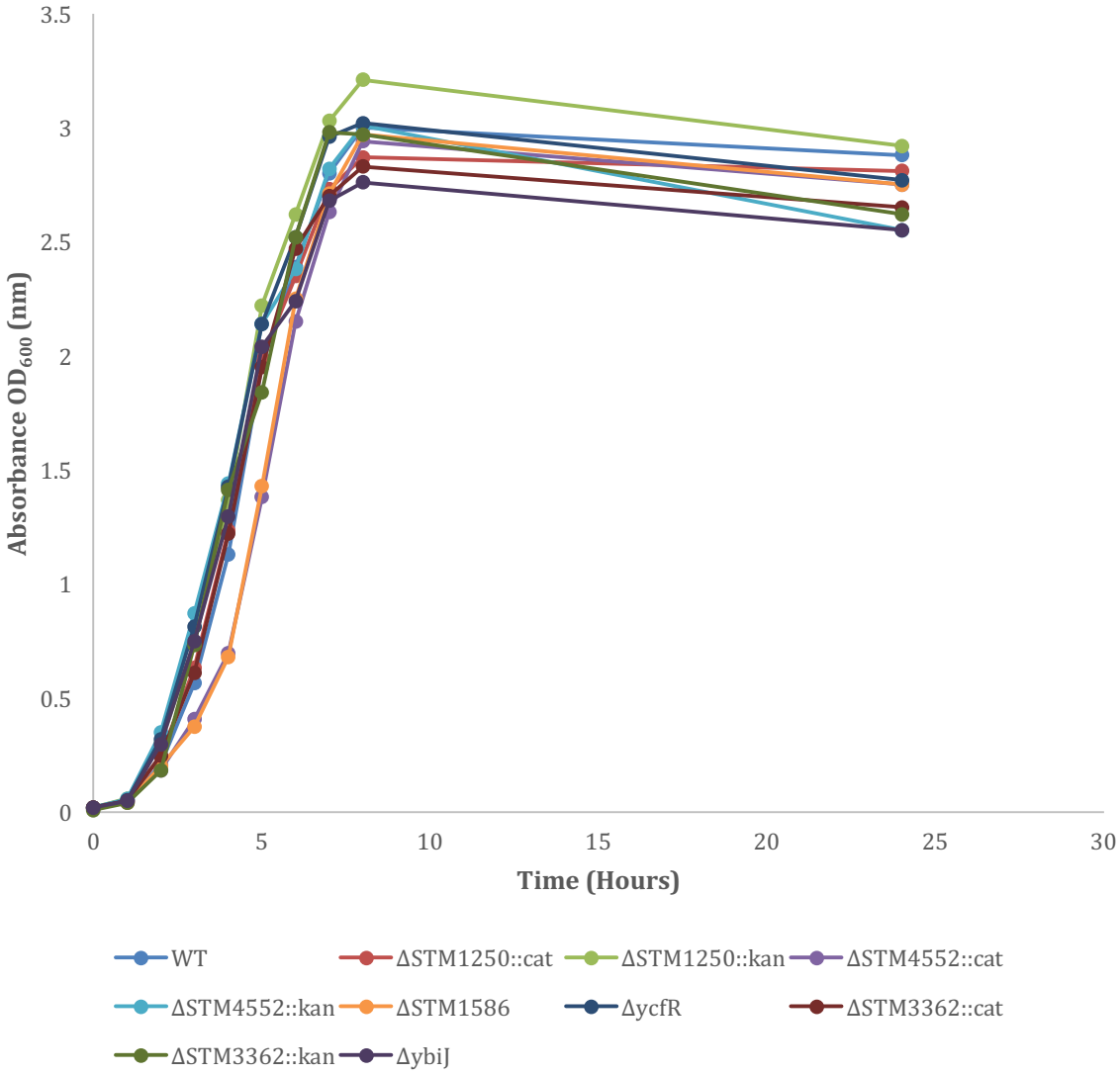


Figure: 22 Growth of WT and single mutants in LB media. Growth of WT, Δ*STM1250,* Δ*STM4552,* Δ*STM1586,* Δ*STM3362,* Δ*ycfR and* Δ*ybiJ* and Δ*STM3362* under aerobic conditions in 50ml LB media. Cultures were grown for 24 hours at 37°C, aliquots were removed and the OD_{600} read every hour.

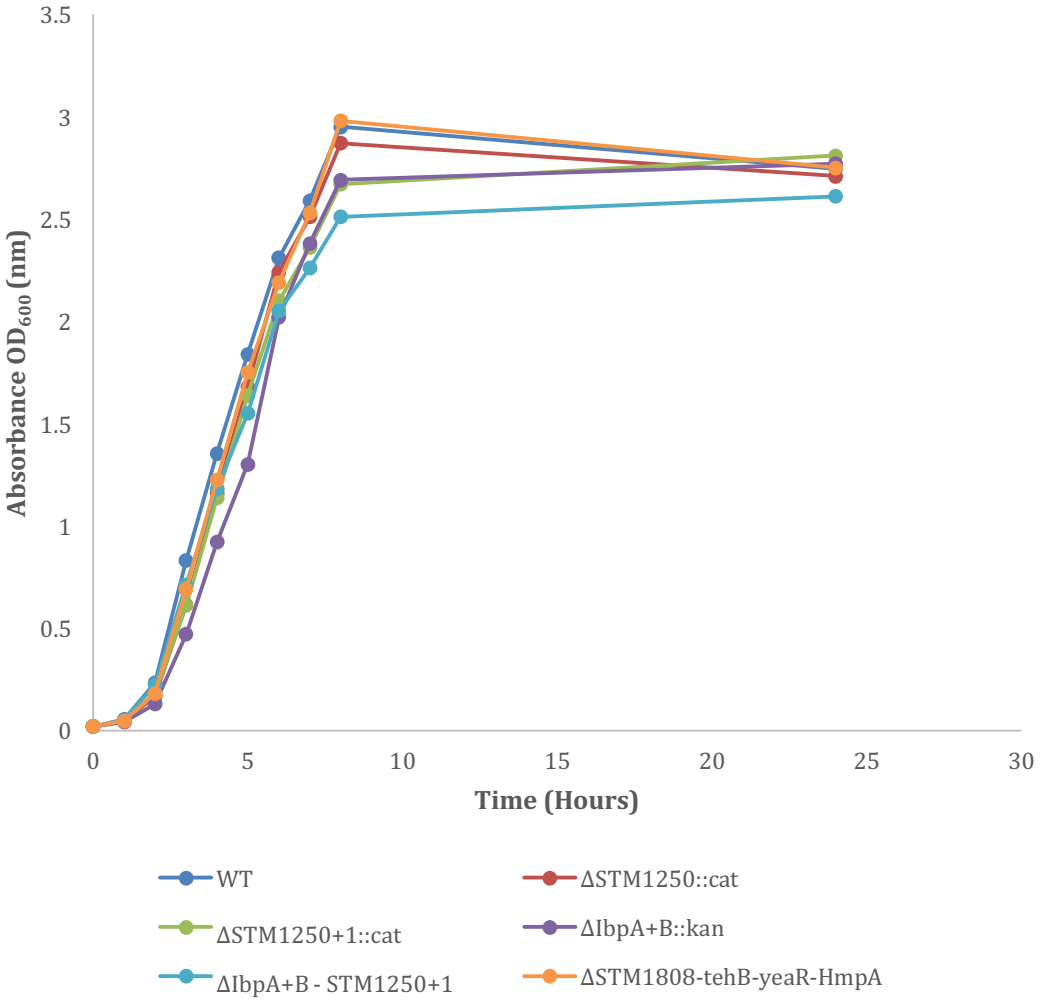


Figure 23: Growth of WT and multiple mutants in LB media. Growth of WT, Δ*STM1250,* Δ*STM1250-STM1251,* Δ*IbpA-IbpB,* and Δ*STM1808-tehB-yeaR-HmpA* were under aerobic conditions in 50ml LB media. Cultures were grown for 24 hours at 37°C, aliquots were removed and the OD_{600} read every hour.

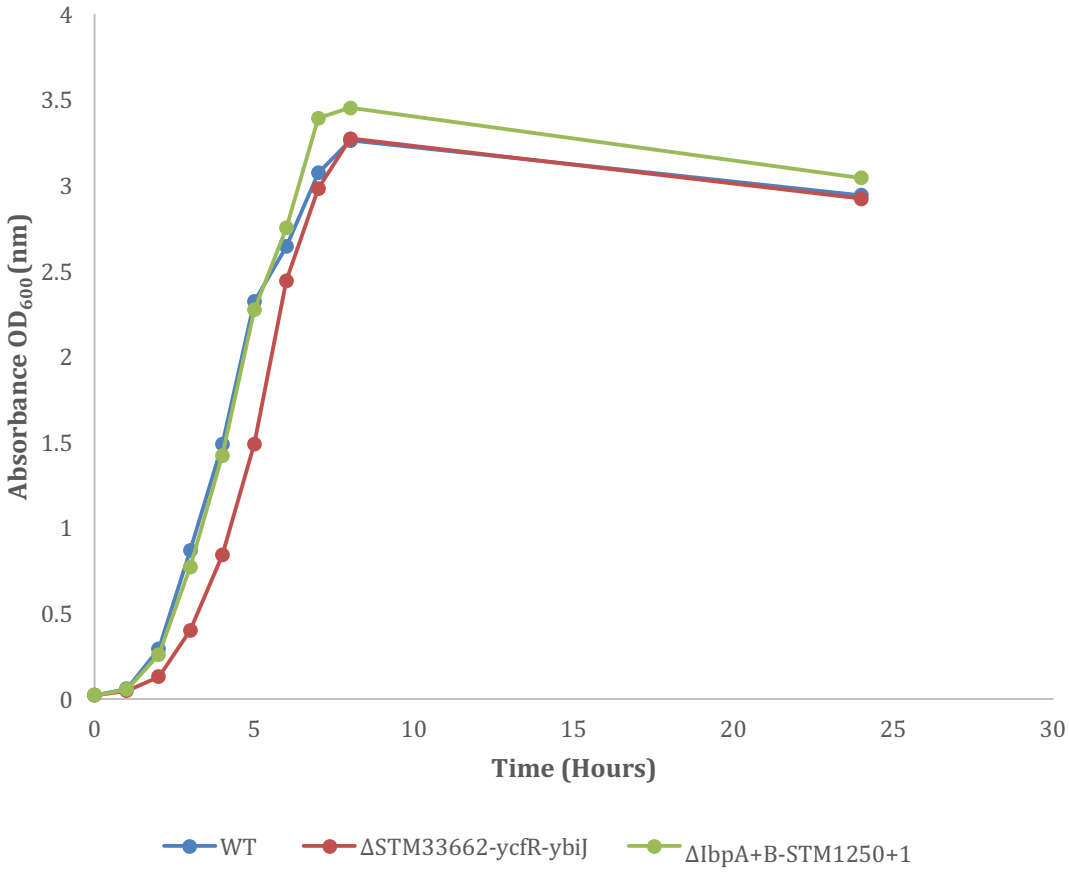


Figure 24: Growth of WT, triple and quadruple mutants in LB media. Growth of WT, *ΔIbpA-IbpB-STM1250-STM1251* and *ΔSTM3362-ycfR-ybiJ*-were under aerobic conditions in 50ml LB media. Cultures were grown for 24 hours at 37°C, aliquots were removed and the OD_{600} read every hour.

3.4.2 Single Mutants are no more sensitive to nitrosative stress than WT *S.* Typhimurium

Single mutants and the WT strain were exposed to 12.5mM DetaNonoate as a source of nitric oxide under both aerobic and anerobic conditons. The quadruple mutant *ΔSTM1808-yeaR-tehB-hmpA* which has previously been shown by the Rowley lab to display extreme sensitivity to nitrosative stress was used to demonstrate the functionality of the assay. Single mutants did not appear to be more sensitive to nitrosative stress than the WT strain under either aerobic or anaerobic conditons (Figure 25, Figure 26).

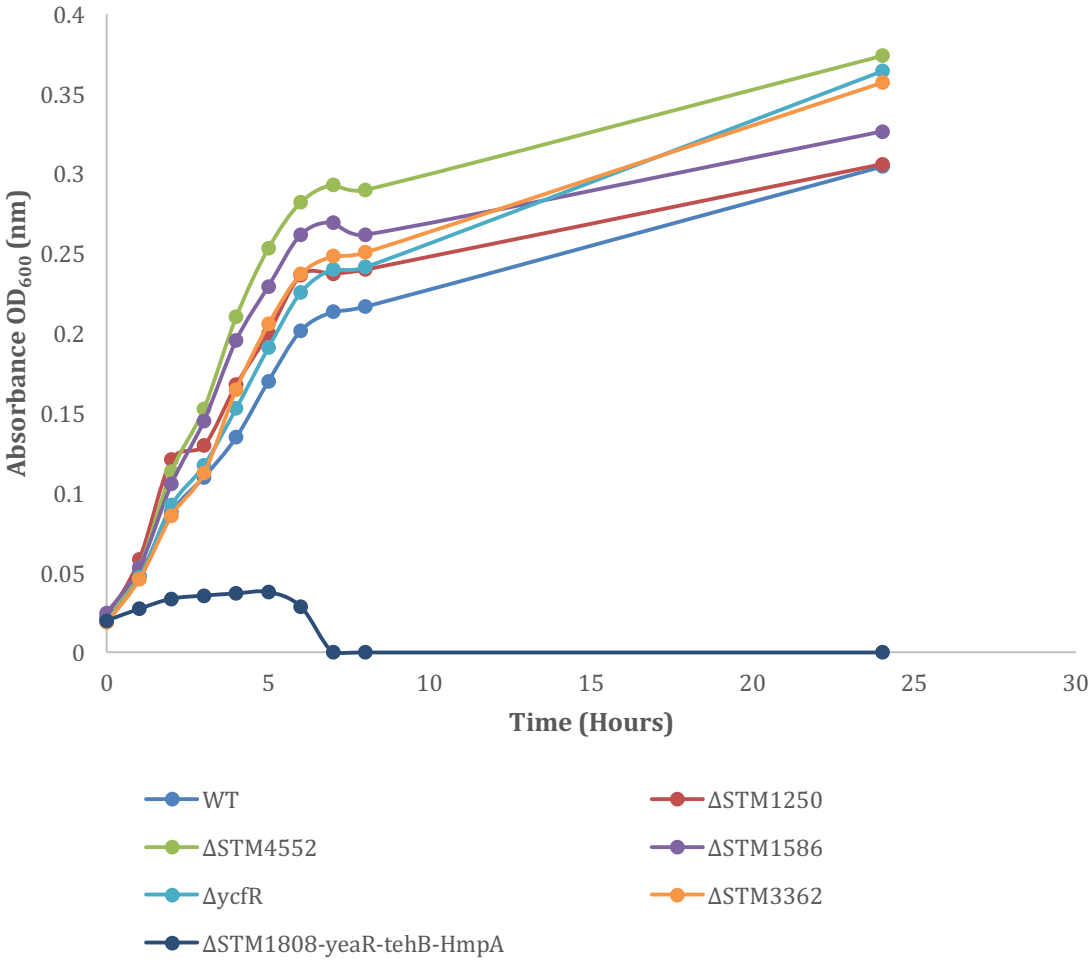


Figure 25: Sensitivity of WT and single mutants to NO under aerobic conditions. The WT strain and mutant strains Δ*STM1250,* Δ*STM4552,* Δ*STM1586,* Δ*ycfR* and Δ*STM3362* were grown aerobically in 96 well plates at 37°C with LB and 12.5mM DetaNONOate. OD readings were taken every hour using a plate reader at 600nm.

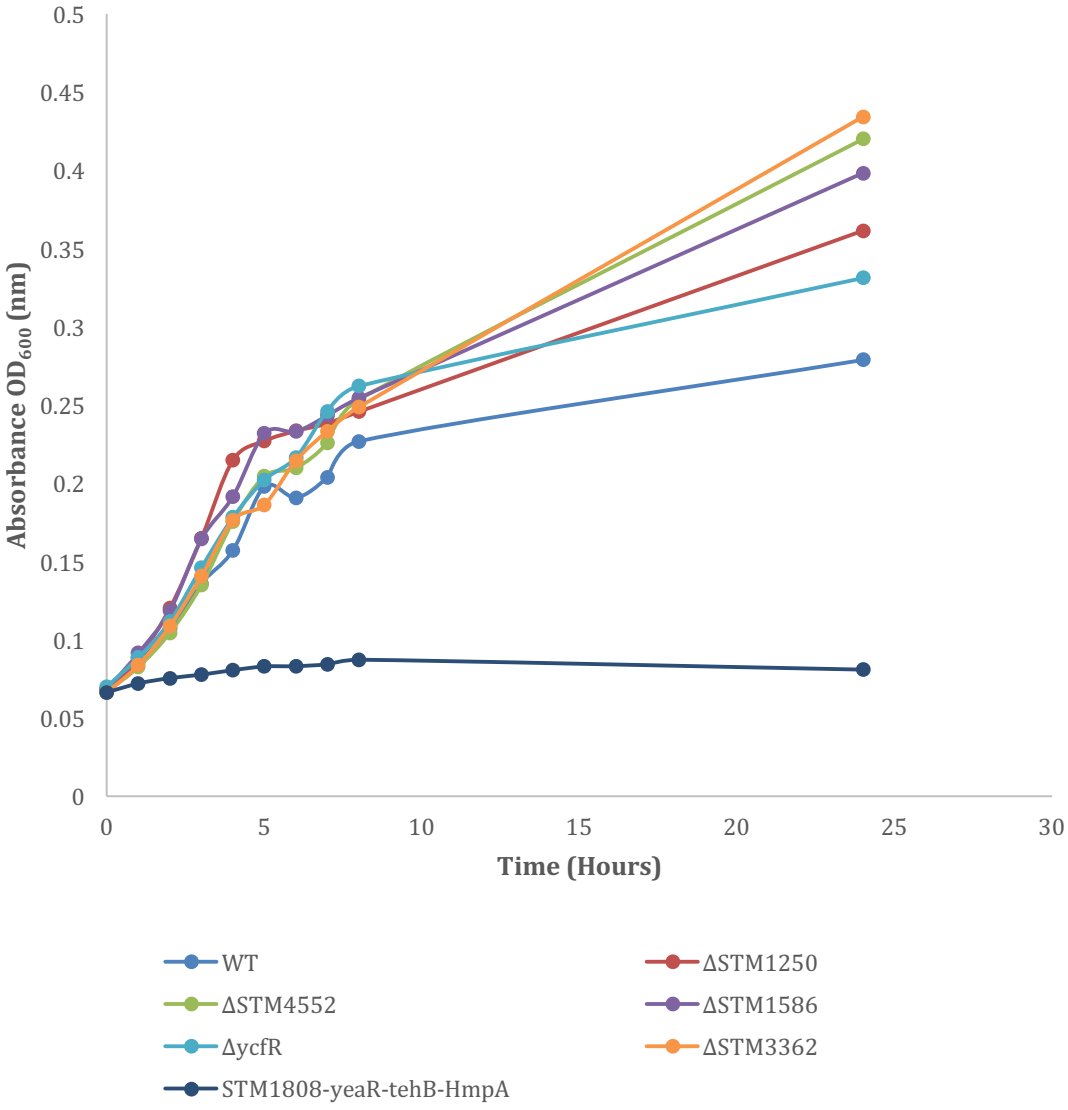


Figure 26: Sensitivity of WT and single mutants to NO under anaerobic conditions. The WT strain and mutant strains Δ*STM1250,* Δ*STM4552,* Δ*STM1586,* Δ*ycfR* and Δ*STM3362* were grown anaerobically in 96 well plates at 37°C with LB and 12.5mM DetaNONOate. OD readings were taken every hour using a plate reader at 600nm.

3.4.3 Δ*STM1250-STM1251,* Δ*ibpA-ibpB* and Δ*ibpA-ibpB-STM1250-STM1251* are more sensitive to nitrosative stress than WT *S.* Typhimurium

Multiple mutants and the WT strain were exposed to 12.5 mM DetaNonoate as a source of nitrosative stress under both aerobic and anerobic conditons. Again, the quadruple mutant *ΔSTM1808-yeaR-tehB-hmpA* was used to demonstrate the functionality of the assay. Multiple mutants appeared to be more sensitive to nitrosative stress than the WT strain under both aerobic, and particularly, anaerobic conditons. Interestingly survival and growth were most affected in the *ΔibpA-ibpB* double mutant rather than the quadruple *ΔibpA-IbpB-STM1250-STM1251* (Figure 27, Figure 28).

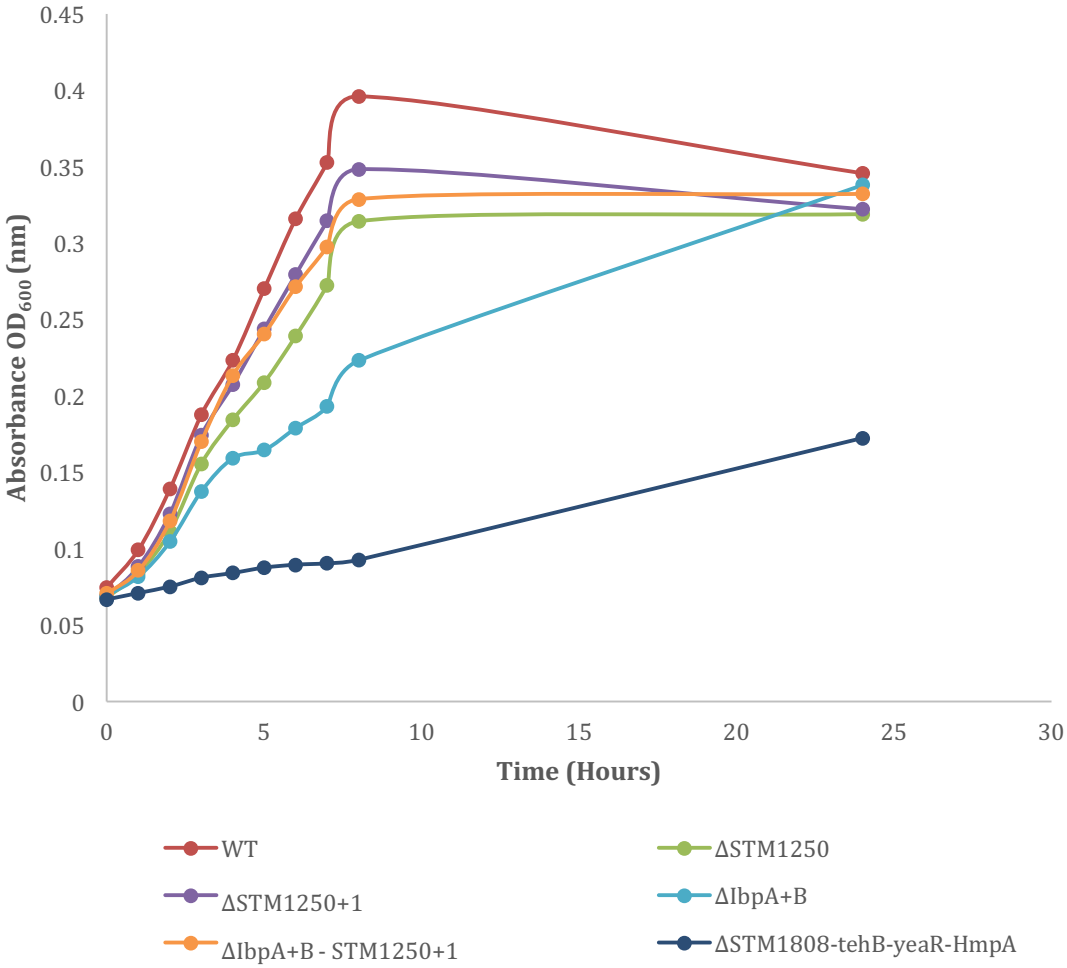


Figure 27: Sensitivity of WT and multiple mutants to NO under aerobic conditions. The WT strain and mutant strains, Δ*STM1250-STM1251,* Δ*ibpA-ibpB* and Δ*ibpA-ibpB-STM1250-STM1251* were grown aerobically in 96 well plates at 37°C with LB and 12.5mM DetaNONOate. OD readings were taken every hour using a plate reader at 600nm.

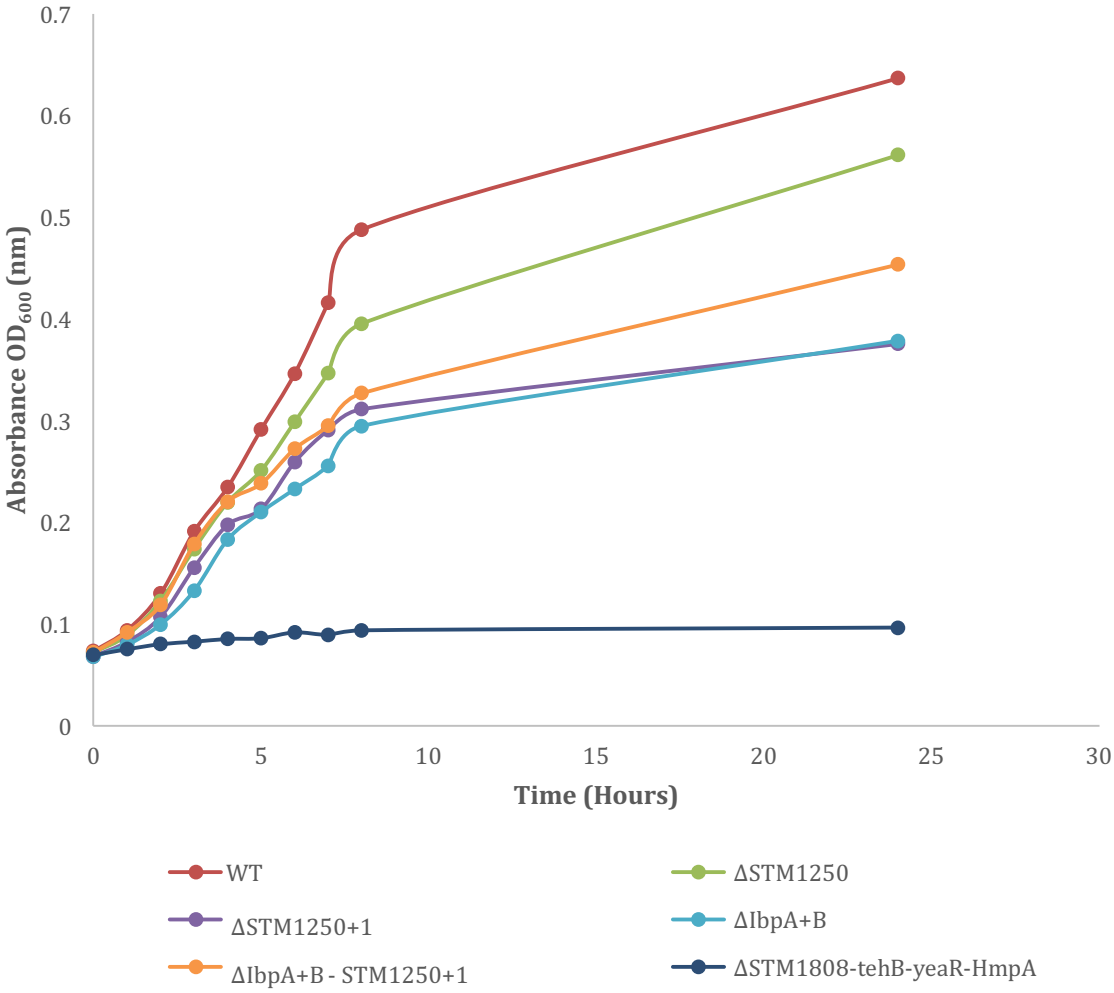


Figure 28: Sensitivity of WT and multiple mutants to NO under anaerobic conditions. The WT strain and mutant strains Δ*STM1250-STM1251,* Δ*ibpA-ibpB* and Δ*ibpA-ibpB-STM1250-STM1251* were grown anaerobically in 96 well plates at 37°C with LB and 12.5mM DetaNONOate. OD readings were taken every hour using a plate reader at 600nm.

3.4.4 ΔSTM1250-STM1251, ΔibpA-ibpB, ΔibpA-ibpB-STM1250-STM1251 and ΔSTM3362-ycfR-ybiJ are more sensitive to oxidative stress than WT S. Typhimurium

Multiple mutants and SL1344 WT strain were exposed to hydrogen peroxide as a source of oxidative stress. Mutiple mutants, Δ*STM1250-STM1251,* Δ*ibpA-ibpB* and Δ*ibpA-ibpB-STM1250-STM1251* all appeared to be more sensitive to oxidative stress than the WT strain, particularly the IbpA-IbpB double mutant (Figure 29).

Hydrogen peroxide susceptibility assays were also performed with the triple mutant, Δ*STM3362-ycfR-ybiJ,* quadruple mutant, Δ*ibpA-ibpB-STM1250-STM1251,* and the WT strain at concentrations of 0mM and 4mM H_20_2. As shown in Figure 30, survival of both mutants is impaired in comparison to WT *S*. Typhimurium, with Δ*ibpA-ibpB-STM1250-STM1251* being the most severely affected. This difference in survival is most apparent in the first 3 hours, and by 8 hours all strains have been eradicated by oxidative stress. These results suggest that Δ*ibpA-ibpB-STM1250-STM1251* and Δ*STM3362-ycfR-ybiJ* are both more sensitive to oxidative stress than WT *S.* Typhimurium SL1344.

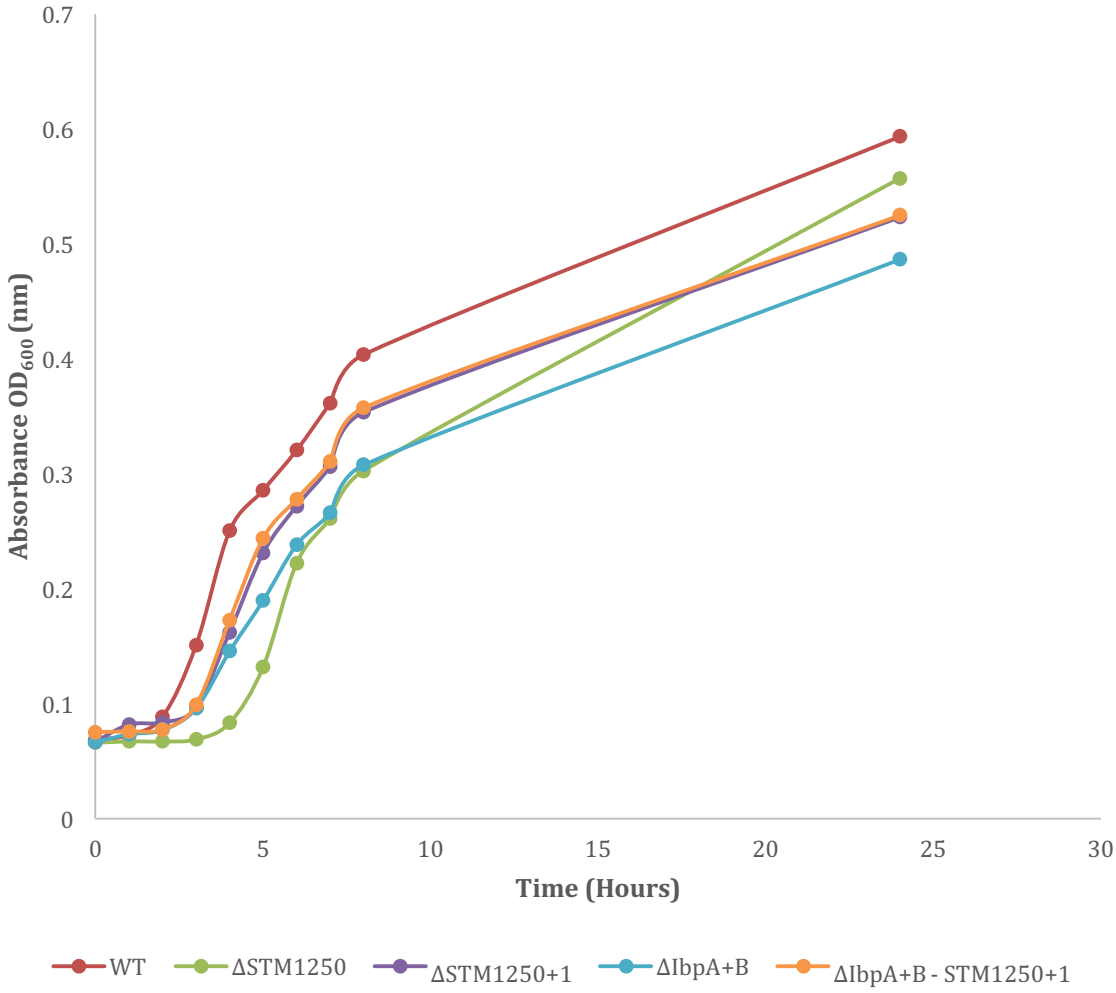


Figure 29: Sensitivity of WT and multiple mutants to H_2O_2. The WT strain and mutant strains Δ*STM1250-STM1251,* Δ*ibpA-ibpB* and Δ*ibpA-ibpB-STM1250-STM1251* were grown in 96 well plates at 37°C with LB and 3mM H_20_2. OD readings were taken every hour using a plate reader at 600nm.

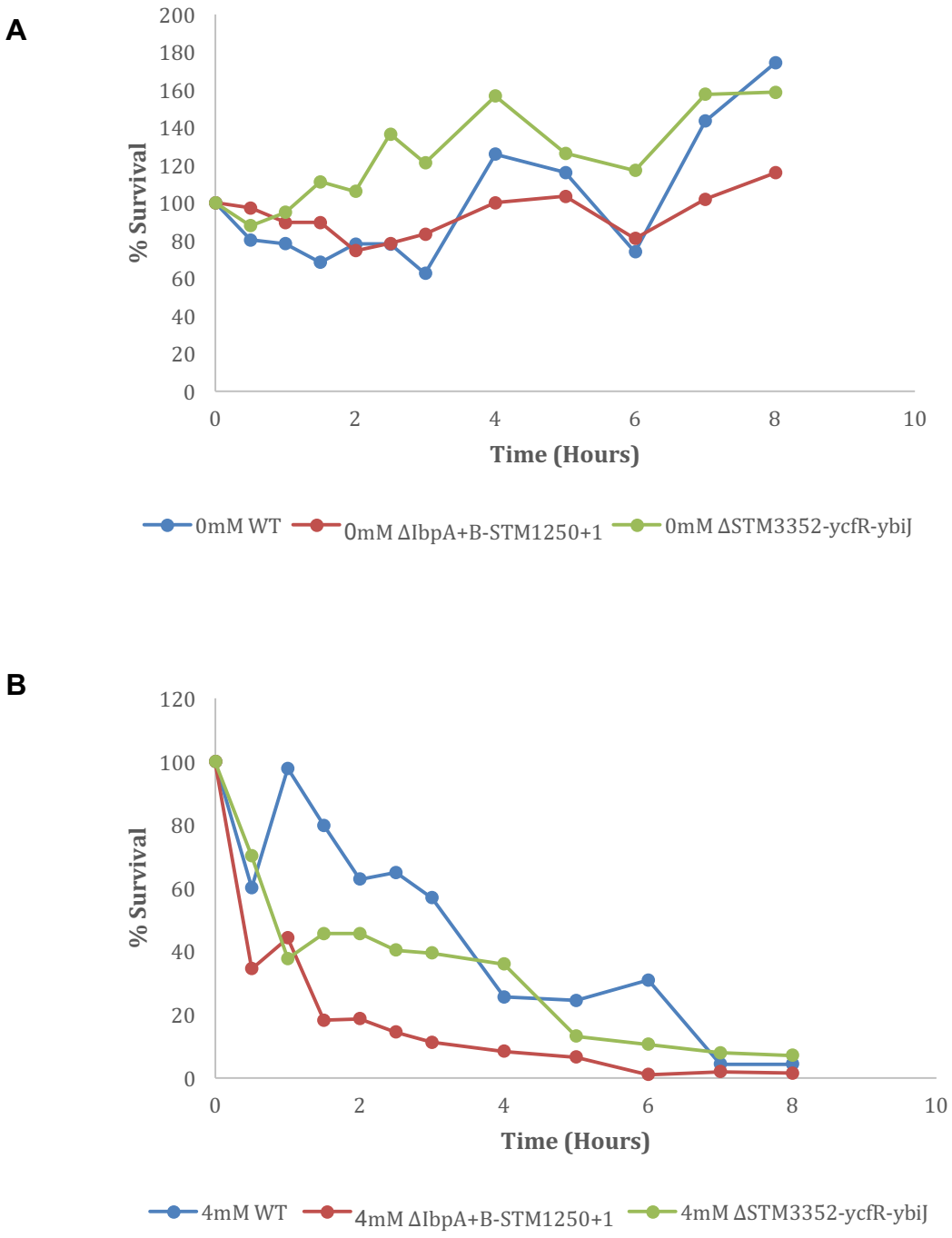
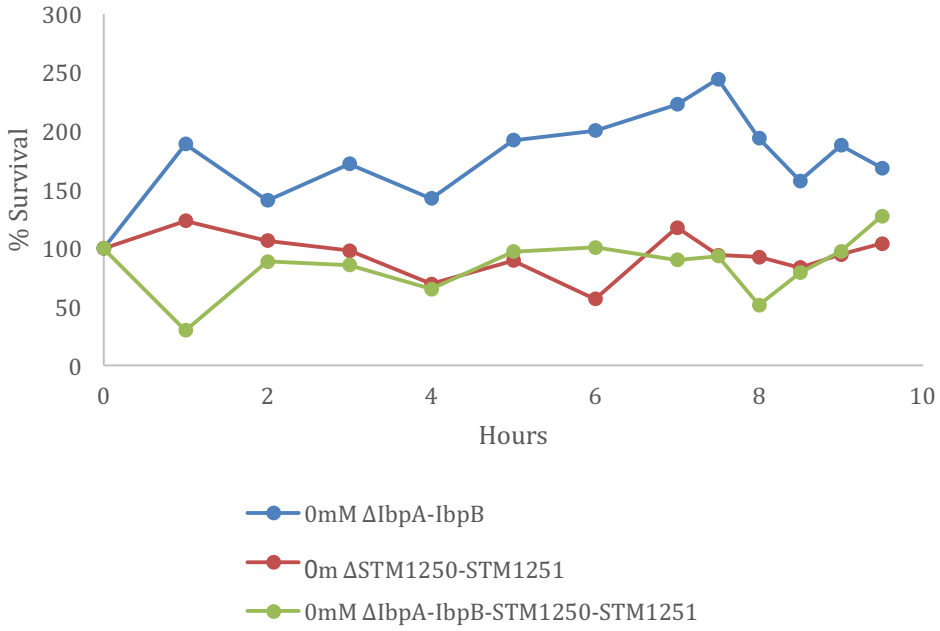


Figure 30: Susceptibility of WT and ΔibpA-ibpB-STM1250-STM1251 and ΔSTM3362-ycfR-ybiJ to H_2O_2. Strains were grown in either 0mM or 4mM H_20_2 in M9 minimal media without glucose or nitrate for 8 hours at room temperature. Aliquots were removed at regularly timed intervals, diluted in PBS and plated on LB agar for quantification of colony-forming units.

3.4.5 The double mutant Δ*ibpA-IbpB* displays the greatest senstivity to NO

To explore the respective contributions of the homologues IbpA & IbpB, and STM1250 & STM1251 to the NO sensitivity of the quadruple mutant a similar assay was performed. In this assay DetaNonoate agin was used as a source of nitrosative stress, with strains exposed to 0mM and 7.5mM DetaNonoate Results shown in Figure 31 indicate that the double mutant *ΔibpA-IbpB* is the strain effected most by nitrosative stress, whilst the double mutant *ΔSTM1250-STM1251* is slightly less susceptible.

A



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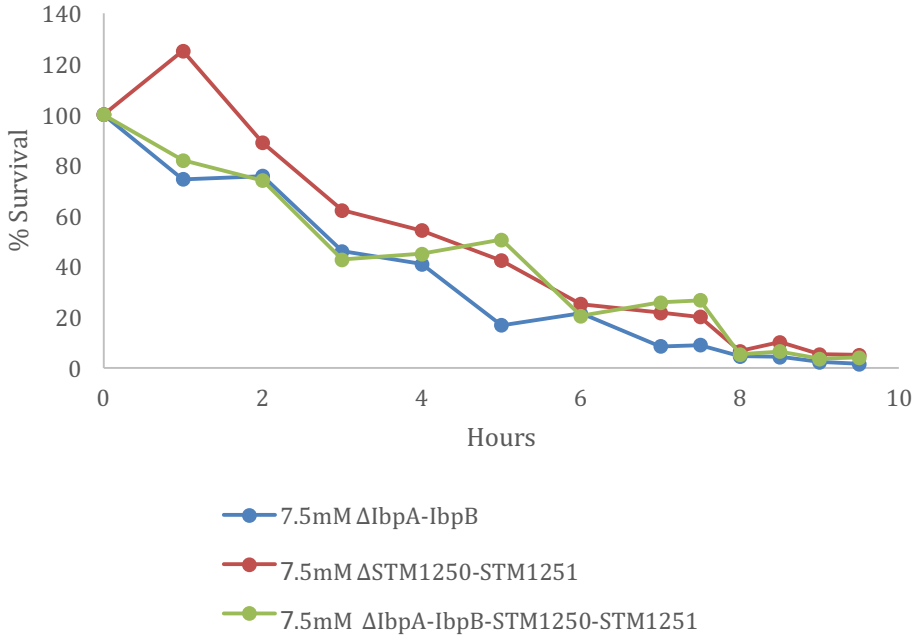


Figure 31: Susceptibility of ΔibpA-ibpB-STM1250-STM1251, ΔibpA-ibpB and ΔSTM1250-STM1251 to DetaNONOate. Strains were grown in either 0mM or 7.5mM DetaNONOate in M9 minimal media without glucose or nitrate for 20 hours at room temperature. Aliquots were removed at regularly timed intervals, diluted in PBS and plated on LB agar for quantification of colony-forming units.

3.5 Discussion

Whilst three main NO-consuming systems, Hmp, NorVW and NrfA, have been characterised, it is thought that other yet-unelucidated mechanisims may contribute to NO stress resistance and detoxification in *S.* Typhimurium. This has been indicated by studies showing that *S.* Typhimurium strains are able to survive anaerobic nitrosative stress even when functional *hmpA, norV* and *nrfA* are abrogated (Mills *et al*, 2008; Torres *et al*, 2016).

This chapter aimed to further investigate this possibility on the basis of preliminary data from the Rowley laboratory which showed up-regulation of various *S.* Typhimurium genes in response to nitrosative stress. To address whether these genes were involved in *Salmonella* nitrosative or oxidative stress response, deletion mutant strains were constructed and exposed to either oxidative stress (hydrogen peroxide) or nitrosative stress (detaNONOate) at physiologically relevant levels.

Construction of both single and multiple mutants by λ Red Recombination was largely successful. The exception to this was a mutant strain of *STM2804*, which was attempted numerous times but failed to yield a confirmed mutant following the phage transduction stage. The study therefore moved forward with the single mutant strains*, ΔSTM4452, ΔSTM1250, ΔSTM1586, ΔSTM3362, ΔybiJ, ΔycfR* and the multiple mutant strains, *ΔibpA-ibpB, ΔSTM1250-STM1251, ΔibpA-IbpB-STM1250-STM1251 and ΔSTM3362-ycfR-ybiJ.* Of the above listed genes, *STM4452, STM1250, STM1586, ycfR, ybiJ* and *ibpA* were investigated due to their up-regulation in the microarray. YcfR was of particular interest due to its apparent role in chlorine stress resistance

in *S.* Typhimurium and as a multiple stress resistance protein in E. *coli* (Deng *et al*, 2011; Salazar *et al*, 2013). Strains including mutations in *ibpB* and *STM1251* were also created due to the homologous nature of ibpA and ibpB, and STM1250 and STM1251, respectively. This was to ensure that resulting phenotypes were not masked by genetic redundancy.

Studies have indicated a role for *STM3362* in resistance to stressors such as H_2O_2, chlorine and cadmium (Eletsky *et al*, 2014; Lee *et al,* 2010; Wang *et al*, 2010). As such a mutant strain of this gene was created and utilised to further construct the triple mutant, ∆*STM3362-ycfR-ybiJ.* This triple mutant was of interest due to the existence of a DUF1471 domain in all three of these proteins. The suggested involvement of proteins containing the DUF1471 domain in stress response invited examination of these genes, both individually and collectively, in the *Salmonella* response to NO and H_2O_2.

Growth curves in nutrient rich media performed prior to stress sensitivity assays demonstrated that single mutant growth was equivalent to the isogenic parent strain, SL1344. Identical growth curves performed with the multiple mutants confirmed similar results, showing no growth deficiencies in comparison to the WT. Results achieved for subsequent assays were therefore uninfluenced by any inherent growth defects within mutant strains.

Despite preliminary microarray results showing the up-regulation of *STM1250, STM4552, STM1586,* and *ycfR* in response to 40mM aqueous NO, sensitivity assays performed in this study failed to demonstrate increased sensitivity of mutant strains of these genes to nitrosative stress. Similarly, Δ*STM3362*,

containing a DUF1471 domain, was no more susceptible to NO killing than the WT strain. Such results were attained under both anaerobic and aerobic conditions, consequently leading to the conclusion that none of these genes appeared vital in the nitrosative response of *S.* Typhimurium in either oxic or anoxic environments.

In contrast, multiple mutants displayed increased sensitivity to nitrosative stress under both aerobic and anaerobic conditions in comparison to WT *S.* Typhimurium. Δ*ibpA-ibpB-STM1250-STM1251,* Δ*ibpA-ibpB* and Δ*STM1250-STM1251* all showed decreased growth in the presence of NO. Similarly, when challenged with oxidative stress these multiple mutants again demonstrated reduced growth in comparison to SL1344.

Unexpectedly, the double mutant Δ*ibpA-ibpB* showed greater sensitvity to nitrosative and oxidative stress than the quadruple mutant Δ*ibpA-ibpB-STM1250-STM1251.* It is perhaps possible that the deletion of all four genes, and thus both homologue pairs of similar function, leads to a compensatory response in *S.* Typhimurium by which the bacterium responds to the deletion to mitigate its negative impact on survival. This would be an example of genetic redundancy, where genes of duplicate function are able to compensate should one fail, allowing for greater adaptibility and robustness of the organism (Nowak *et al*, 1997). Such a compensatory mechanism would explain why a deletion of *ibpA* and *ibpB* resulted in a more stress susceptible strain than Δ*ibpA-ibpB-STM1250-STM1251,* as it retained the *ibpA* and *ibpB* homologues *STM1250* and *STM1251* which may perform similar, yet less efficient, functions in *Salmonella* stress response. This would make sense in

that *Salmonella* alone posses these homologues, whereas the similar microoganism *E. coli* functions competently with solely *ibpA* and *ibpB*. Currently it is unclear why *STM1250* and STM1251 are present in *Salmonella* alone.

Whilst results showed that the double mutant Δ*ibpA-ibpB* was more susceptible to nitrosative stress under aerobic conditons than either Δ*ibpA-ibpB-STM1250-STM1251* or Δ*STM1250-STM1251,* under anaerobic conditons, Δ*ibpA-ibpB* and Δ*STM1250-STM1251* displayed near identical susceptibility to NO. It may therefore be proposed that *STM1250* and *STM1251* serve a more considerable contribution to NO resistance under anoxic or low oxygen conditons than they do in aerobic environments, or that *IbpA* and *IbpB* are less vital in the anaerobic nitrosative stress response than under aerobic conditons. However far more experimentation would be required before either supposition could be substantiated.

Susceptibility assays described and implemented by Testerman *et al*, were modified to investigate the impact of both oxidative and nitrosative stress on mutant strains. This additional testing with susceptibility assays showed that both *ΔibpA-ibpB-STM1250-STM1251* and the triple mutant, Δ*STM3362-ycfR-ybiJ* were sensitive to oxidative stress. When challenged with 4mM H_2O_2, both mutant strains showed decreased survival than that demonstrated in the absence of oxidative stress. Furthermore, both *ΔibpA-ibpB-STM1250-STM1251 and* Δ*STM3362-ycfR-ybiJ* were more susceptible to oxidative killing than the isogenic parent strain at the same concentration of hydrogen peroxide, with *ΔibpA-ibpB-STM1250-STM1251* being the most affected.

Δ*STM3362-ycfR-ybiJ* was of interest due to the presence of DUF1471, exclusive to Enterobacteriaceae and commonly found in proteins involved in extracellular stress responses (Eletsky *et al*, 2014). The susceptibility of Δ*STM3362-ycfR-ybiJ* to oxidative stress suggests that these genes may indeed play minor roles in the oxidative stress reponse of *S.* Typhimurium.

NO susceptibity assays were also performed on *ΔibpA-ibpB-STM1250-STM1251, ΔibpA-ibpB* and *ΔSTM1250-STM251* using physiologically revelant levels of nitric oxide provided by 7.5mM of deta NONOoate (Hussain *et al*, 2014). Results similarly showed that these mutant strains were sensitive to NO killing. Whilst all strains were effected, based on strain survival in the absence of NO, *ΔibpA-ibpB* again appeared to be most susceptible to nitrosative stress. Similarly, the second most susceptible was the quadruple mutant *ΔibpA-ibpB-STM1250-STM1251*, whilst *ΔSTM1250-STM251* showed slightly higher survival. Results of this assay therefore complemented those achieved for these multiple mutants in sensitivity experiements.

It is possible to conclude therefore that results from this study suggest a role for various *S.* Typhimiurium genes in oxidative and nitrosative stress survival other than *hmpA*, *norVW* and *nrfA*.

Though single mutants did not display increased NO sensitivity, is is plausible that with the main NO detoxficiation protein HmpA still functional and active, the deletion phenotype of these genes were not signficant enough to effect *Salmonella* survival. Whilst such results suggest that their influence is

minimal, it does not eliminate the possibility of their involvement in NO detoxification entirely.

In contrast, based on results of susceptibility assays Δ*STM3362-ycfR-ybiJ* may be involved in the oxidative stress tolerence of *S.* Typhimurium, while mutant strains *ΔibpA-ibpB, ΔSTM1250-STM1251* and *ΔibpA-ibpB-STM1250-STM1251* all demonstrate increased sensitivity to both NO and H_2O, therefore suggesting a role for these genes in NO detoxification and the oxidative stress response. IbpA and IbpB in particular appear to have a signficant effect on strain survival of these stressors which bears further investigation. These two small heat shock proteins share a 48% sequence identity and are known to be upregulated in *E. coli* and *Salmonella* following heat stress, where they act to prevent protein aggregation (Matuszewska *et al*, 2005; Laskowska *et al*, 1996; Carroll *et al*, 2016). It has been previously proposed that IbpA and IbpB are also involved in some capacity in oxidative stress tolerence in *E. coli*, with bacteria overexpressing *ibpA/B* showing increased resistance to superoxide stress (Kitagawa *et al*, 2000). Further *in vitro* study by the same group showed that IbpA and IbpB were capable of supressing the inactivation of enzymes by H_2O_2 and potassium superoxide (Kitagawa *et al*, 2002). Strains of *E. coli* deficient in *ibpA/B* also show higher sensitivity to superoxide radicals produced by potassium tellurite (Perez *et al*, 2007). Furthermore, it appears that under aerobic conditons IbpA/B protect cells from copper-induced stress, with Δ*ibpA/B* strains showing increased sensitivity to oxidative damage from copper ions (Matuszewska *et al*, 2008). More recently, non-pathogenic *E. coli* inside macrophages were found to upregulate *ibpA/B* in response to ROS, where *ibpA/B* appeared to then protect *E. coli* from ROS killing (Goeser *et al*,

2015). Although less is known about *ibpA/B* expression in response to NOS, the results of this study suggest that IbpA and IbpB may play a minor role in both oxidative and nitrosative stress tolerence.

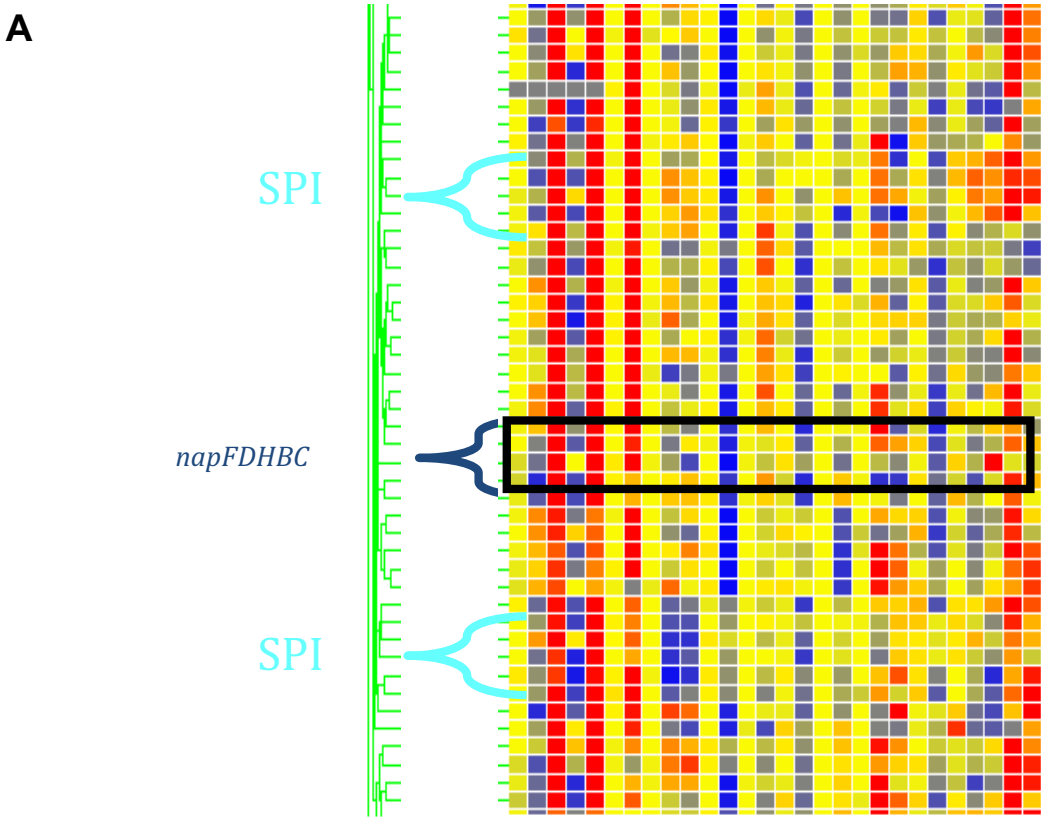
CHAPTER 4: Regulation of the *napF* operon by the SPI-1 transcriptional activator HilA

4.1 Introduction

As previously discussed, the *Salmonella HilA* transcriptional regulator is the well-characterised activator of SPI-1 (Baxter & Jones, 2015). The genes of SPI-1 are expressed when *Salmonella* reaches the intestinal lumen and associate with the epithelial cells or enterocytes where they are required for invasion of the intestinal epithelium (Fàbrega & Vila *et al*, 2013). SPI-1 encodes a TTSS and effector proteins which lead to distortion of the host cell membrane and ultimately the entry of the bacterium (Martinez *et al*, 2014). As the master regulator of SPI-1 the upregulation of HilA is necessary for the expression of SPI-1 genes and subsequent host-cell invasion (Bajaj *et* al, 1995; Baxter & Jones, 2015). Mechanistically, HilA binds directly to and activates the SPI-1 TTSS promoter and also induces InvF, an AraC-like transcriptional regulator that induces expression of SPI-1 secreted effectors (Lucas & Lee, 2001). HilA itself is a member of the ToxR/OmpR-like family of transcriptional regulators and its expression is induced by several transcriptional factors which include HilC, HilD, RtsA, PhoP, SirA and Fis (De Keersmaecker *et al*, 2005). Environmental cues thought to induce the expression of HilA, and therefore SPI-1, are those found in the host intestinal lumen, including high osmolarity, low pH and anoxic conditons. (Lunderberg *et al*, 1999).

Data obtained from previously performed herarchial cluster analysis by *Alston et al* (unpublished data) showed that expression of SPI genes correlated with 5 *nap* genes, *napFDHBC* (Figure 32A). Furthermore a *S.* Typhimurium *nap* deletion mutant showed decreased invasive ability when compared with SL1344 WT (Alston *et al,* unpublished data; Prior, 2011). Nap is the

periplasmic nitrate reductase of *Salmonella* which is encoded by the NapF operon. Under nitrate-limited conditons it is thought to be the main nitrate reductase in nitrate metabolism, allowing *Salmonella* to survive under anaerobic conditons. Due to its higher-affinity for nitrate it is able to function in concentrations of nitrate too low to support respiration by the membrane-bound nitrate reductase, NarG (Rowley *et al*, 2012). The expression of SPI-1 genes clustered with *nap* expression may indicate that they share a common transcriptional regulator, HilA. Furthermore, the upstream sequence of *napF* was aligned with other known HilA targets, including *invF,* and a potential HilA box was identified (Figure 32B). Moreover, as the *nap* deletion mutant shows an impaired ability to invade epithelial cells, it is possible that these *nap* genes could play a role in *Salmonella* invasion in concert with SPI-1. It is therefore plausible and worth investigation whether SPI-1 and *nap* share a common transcriptional activator in HilA.



B

***HilA* box**

napF	ggta**tgcatc**tcttac
invF	tgat**tgcatcag**gatt
prgH	tatt**ttcatcag**gaat
spi4_H	ggct**tgcatcag**gttt
sicA	taac**tgcatcag**ataa
hilA	tctc**tgcaccag**gata
	TgCAtCAGg

Figure 32: Expression of Nap genes coincides with SPI-1 gene expression. (A) Hierarchical cluster analysis by Alston's group suggests that SPI1 genes cluster with 5 nap genes, napFDHBC. It is a possibility therefore that SPI-1 and Nap may have a co-regulatory mechanism, HilA, and that Nap genes may play a role in invasion. (B) Upon sequence analysis of the *napF* operon a potential sequence was identified which might serve as a *HilA* box, such as found in other *HilA* targets.

4.2 Aims

The aim of this chapter was to determine whether HilA, the transcriptional activator of SPI-1 also acts as an activator of the Nap nitrate reductase. *Salmonella* strains containing a *nap-lacZ* fusion were first grown on tetracycline selective antibiotic resistance plates to confirm they contained the pMP220 plasmid. Following this, β-galactosidase assays were performed in both SPI-1 inducing conditions and plain LB to acess whether SPI-1 induction, and therefore *hilA* activation, corresponded with *nap* induction. Finally, β-galactosidase asssays were performed in high and low nitrate conditons, the latter of which is proposed to induce *napF* expression.

4.3 Methods

4.3.1 β -Galactosidase Asssays

S. Typhimurium strains containing a *nap-lacZ* fusion in WT or a *hilA* deletion background had been previously created. Both strains which contained the pMP220 empty vector were used as negative controls.

Aerobic and anaerobic β-galactosidase assays were carried out in order to ascertain whether HilA is a co-regulator of SPI-1 and Nap nitrate reductase.
β-galactosidase is an enzyme encoded by the *lacZ* gene in the lac operon of *E. coli.* It is a 120kDa protein capable of forming a tetramer that cleaves galactose to produce glucose (Juers *et al*, 2012). The synthetic compound o-nitrophenyl- β-D-galactosidase (ONPG) is a lactose analogue and is also cleaved by β-galactosidase to yield galactose and o-nitrophenol (ONP). ONP is a yellow compound and results in a yellow colour change (Juers *et al*,

2012). β-galactosidase assays function on the principle that when ONPG is in excess to the enzyme the production of o-nitrophenol is proportional to the concentration of β-galactosidase. As a result, measurement of the OD of the yellow solution produced allows enzyme concentration to be determined. Strains of *Salmonella* which contained *lacZ* fused to the *napF* promoter were used in these assays. The acitivity of the *napF* promoter was determined by measurement of β-galactosidase activity, as *napF* activity should be directly proportional to that of β-galactosidase in these strains. β-galactosidase assays were first performed aerobically in plain LB media and then in a high salt LB media (LBS) that induced HilA to promote SPI-1 expression. LBS is therefore known as SPI-1 inducing conditons. β-galactosidase assays were then carried out under anaerobic conditons in M9 minimal media with high nitrate and low glycerol (N^+/G^-) and also conversely, with low nitrate and high glycerol (N^-/G^+). Anaerobic, low-nitrate conditons are thought to be those under which *napF* expression is induced (Rowley *et al,* 2012). The Miller equation (Figure 33) was then used to analyse results and determine *napF* activity.

$$\frac{[(A_{420} - 1.75 \times A_{550}) \times 1000]}{(A_{600} \times \text{time} \times \text{volume of permeabilized cells used})}$$

Figure: 33 The Miller Equation. Used to calculate protein of interest concentration from activity of β-galactosidase.

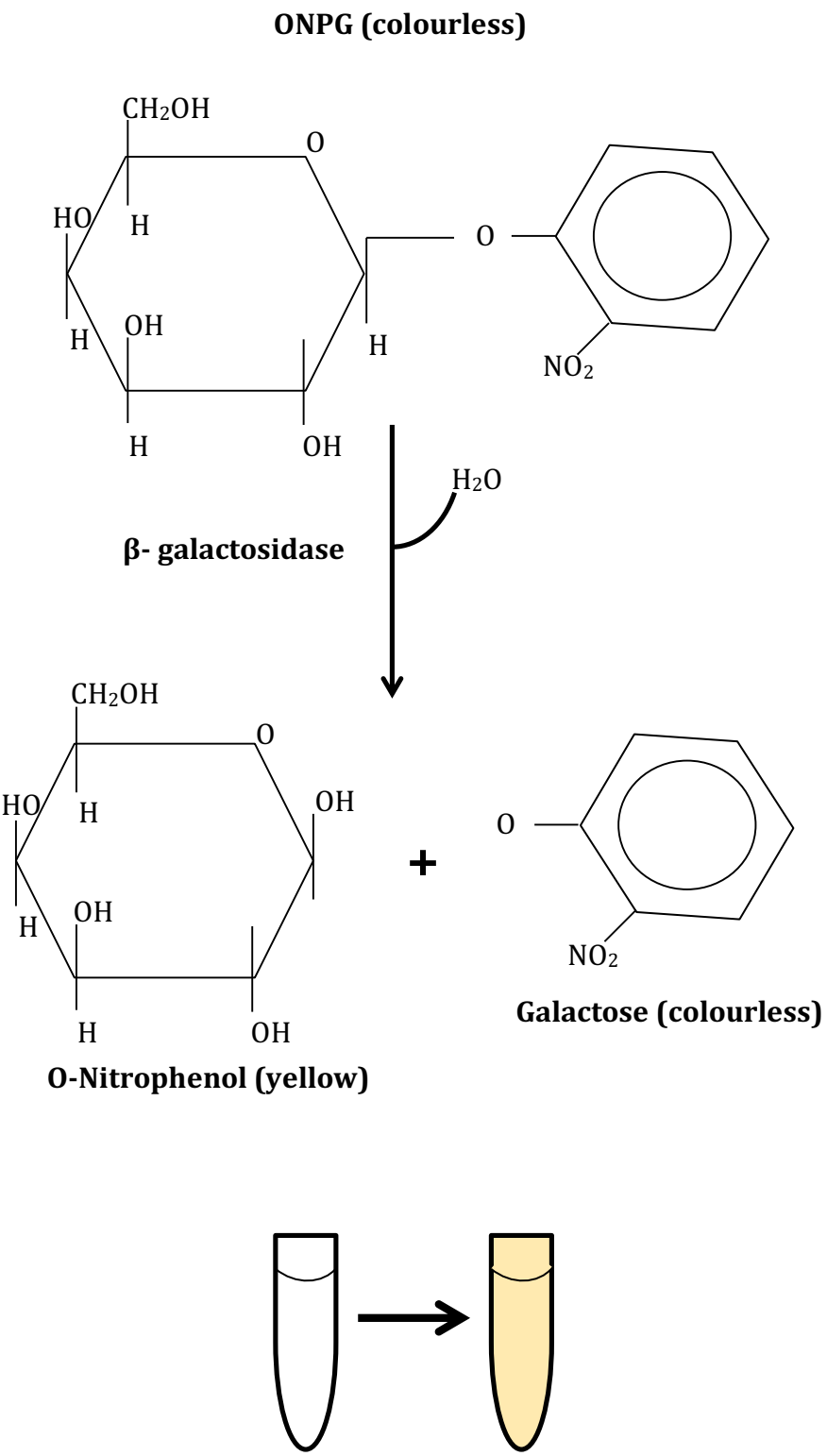


Figure 34: β- galactosidase cleaves ONPG resulting in a visible colour change. ONPG, a colourless, lactose analogue, is cleaved by β-galactosidase enzyme, resulting in ONP, a yellow substrate. This results in a solution colour change from colourless to yellow.

4.4 Results

4.4.1 HilA is not a transcriptional activator of *nap* genes in LBS or LB media.

Figure 35 and 36 show results of aerobic β-galactosidase assays performed in LB and LBS media respectively. Assays performed aerobically in plain LB media (Figure 35) show a 1.25 fold increase in *napF* activity in the *hilA* deletion background compared to when HilA is present. By comparison assays performed aerobically in LBS, SPI-1 inducing conditons, showed no difference in *napF* expression between WT and *hilA* deletion backgrounds (Figure 36). This suggests that HilA is not a transcriptional activator of *napF* under these conditions.

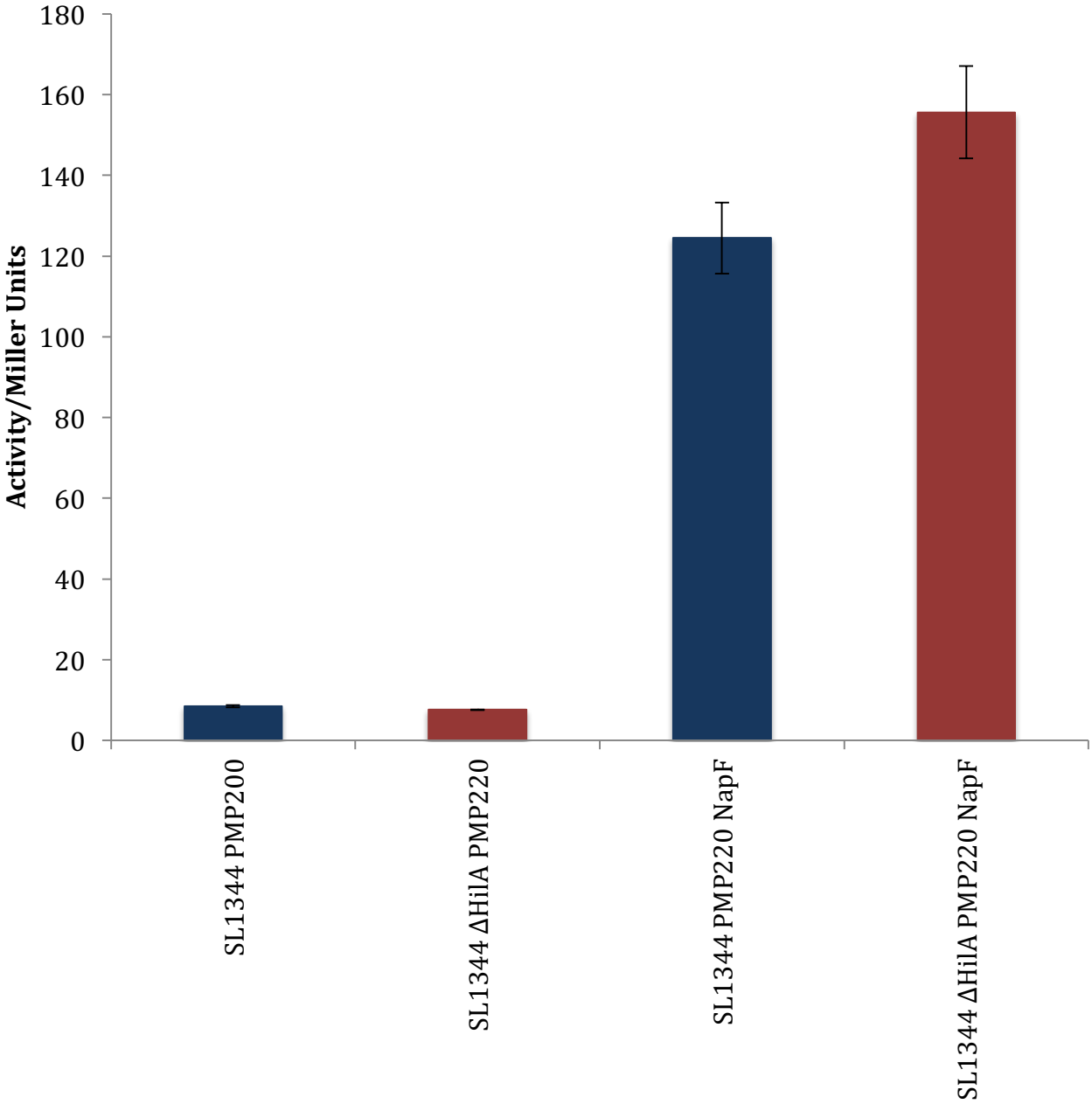


Figure 35: Aerobic Luria Broth β-Galactosidase assay pMP220 with *napF* promoter and empty plasmids. Culture diluted in 2ml Z-buffer was lysed with chloroform and SDS-page prior to the addition of ONPG. The time taken for a yellow colour to develop was recorded and Miller units calculated.

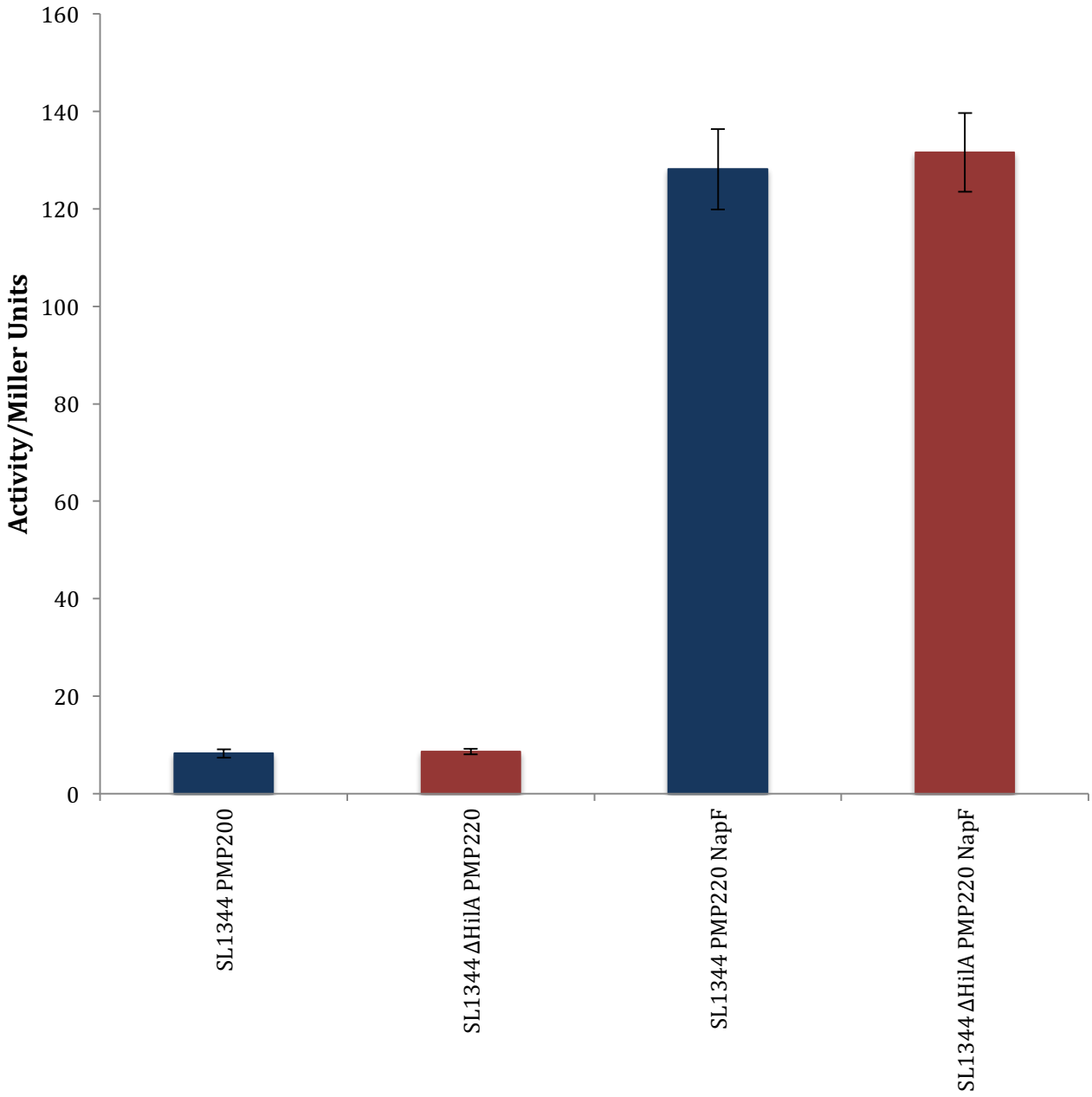


Figure 36: Aerobic high salt Luria Broth β-Galactosidase assay pMP220 with *napF* promoter and empty plasmids. Culture diluted in 2ml Z-buffer was lysed with chloroform and SDS-page prior to the addition of ONPG. The time taken for a yellow colour to develop was recorded and Miller units calculated.

4.4.2 HilA is not a transcriptional activator of *nap* genes in M9 minmal media.

Figure 37 and 38 show results of anaerobic β-galactosidase assays performed in M9 minimal media, N^-/G^+ and N^+/G^- respectively. Anaerobic assays performed in M9 minimal media N^-/G^+, show a 1 fold increase in *napF* activity in the *hilA* deletion background (Figure 37). As did anaerobic assays performed in M9 minimal media N^+/G^- (Figure 37).

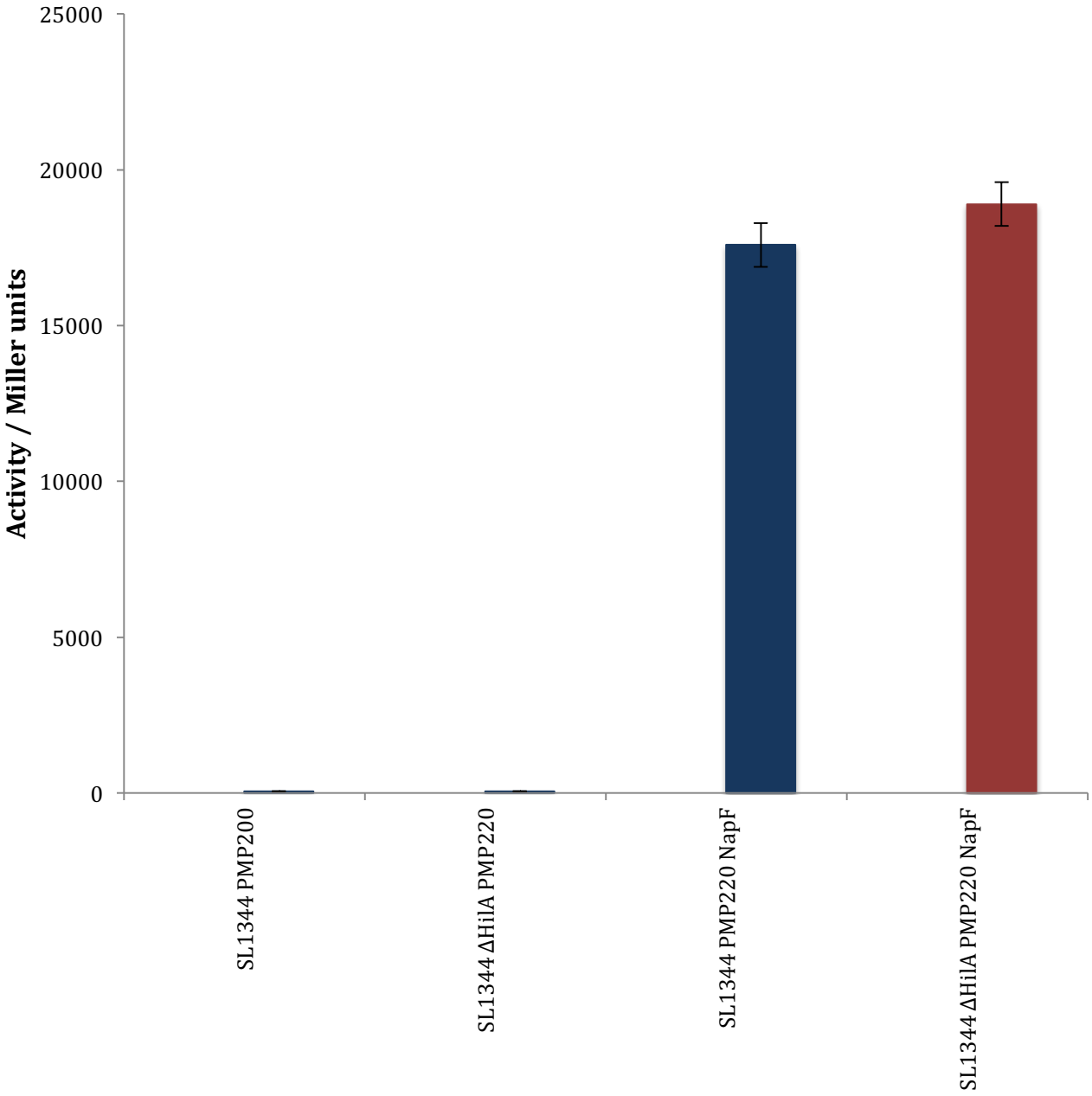


Figure 37: Anaerobic M9 Minimal media with 20mM glycerol, 5mM nitrate, β-Galactosidase assay pMP220 with *napF* promoter and empty plasmids. Culture diluted in 2ml Z-buffer was lysed with chloroform and SDS-page prior to the addition of ONPG. The time taken for a yellow colour to develop was recorded and Miller units calculated.

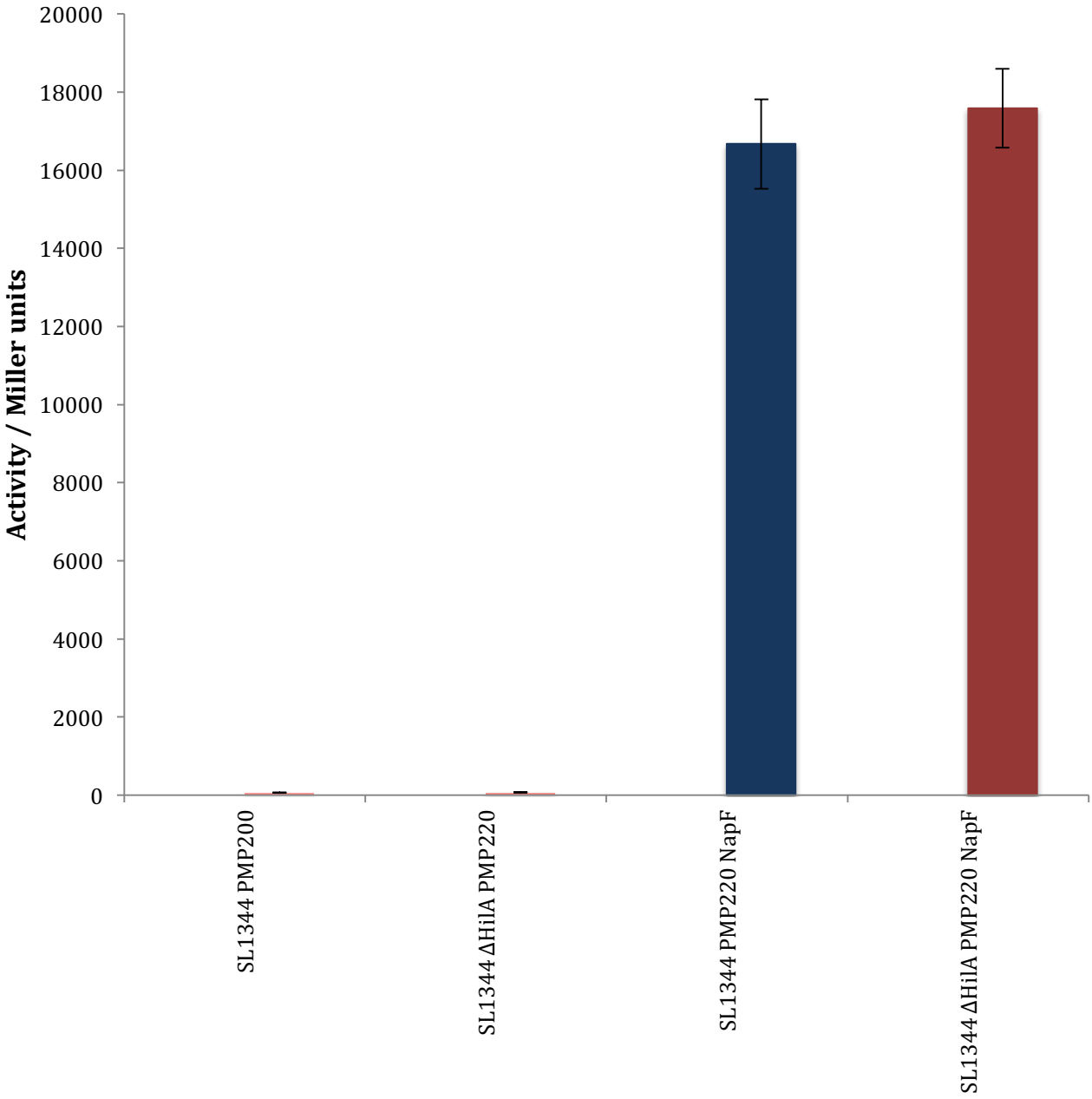


Figure 38: Anaerobic M9 Minimal media 5mM glycerol, 20mM nitrate, β-Galactosidase assay pMP220 with *napF* promoter and empty plasmids. Culture diluted in 2ml Z-buffer was lysed with chloroform and SDS-page prior to the addition of ONPG. The time taken for a yellow colour to develop was recorded and Miller units calculated.

4.5 Discussion

SPI-1, which provides the mechanism by which *S.* Typhimurium invades host epithelial cells, is induced by the transcriptional activator HilA. Unpublished data by Alston *et al* (Figure 32) found that SPI-1 genes are co-expressed with genes of the *nap* operon and furthermore that a *nap* operon deletion mutant displayed a 20-30% reduced ability to invade epithelial cells than the WT. As such it was proposed that *nap* may have a role to play alongside SPI-1 in the early stages of invasion and that HilA may be a transcriptional activator of *nap* as well as SPI-1. The aim of this chapter was to determine whether or not *nap* was induced by HilA under conditions similar to that of the host intestinal lumen where inasion takes place.

Under SPI-1 inducing conditions, there was no signfiicant difference in *napF* expression regardless of whether HilA was present or absent. In fact, a slight increase in *napF* expression in the *hilA* deletion background was observed. Should HilA, which activates SPI-1 under similar conditons, be an activator of *napF* also, it was expected that *n*apF expression would have been markedly increased in the HilA competent background and near negligable when HilA was absent. Therefore the results of this study suggest that HilA is not a transcriptional activator of *napF* expression. Results from assays performed anaerobically in M9 minimal media displayed corresponding results, including the slightly higher activity of the *napF* promoter in the hilA deletion background. Therefore it is possible that HilA may infact exert negative regulation on NapF, as activity of the nitrate reductase appears to be slightly higher in *hilA* deletion backgrounds. This aligns with earlier results from Prior, 2011, who also observed that NapF appeared to undergo negative regulation

by HilA under both aerobic and anaerobic conditions. Combined with the prior analysis from Alston *et al* showing a putative hilA box in the *napF* operon and co-expression with SPI-1 genes, this data seems to suggest the possible negative regulation of *napF* by HilA.

To determine whether HilA does indeed negatively regulate *napF*, *S.* Typhimurium strains containing a mutation to the *hilA* promoter region could be constructed and *napF* activity of these strains determined. ChIP-on-chip and Electrophoretic Mobility Shift Assays (EMSA) could be employed to investigate the interaction of HilA and the *napF* operon *in vivo.* Should such future work confirm that NapF expression is regulated by HilA then further lines of inquiry should examine why such regulation exists and how it benefits *S.* Typhimurium.

CHAPTER 5: General Discussion

As each chapter has already been individually discussed, I here summarize the major outcomes of this study and contextualize these results in the greater body of *Salmonella* research. Additionally I will conclude with suggestions and framework for future investigations.

5.1 Context

5.1.1 *Salmonella* and Global Health

Members of the *Salmonella* genus are bacterial pathogens capable of causing mild to fatal disease in humans and mammals. These diseases are gastroenteritis, more commonly known as food poisoning, and the more severe Typhoid Fever. Current vaccines available for Typhoid offer inadequate solution for countries in which the illness is endemic. Furthermore, recent years have shown a dramatic and concerning increase in strains of antibiotic resistant *Salmonella*. As such there is a prerequisite for research which facillitates the development of novel and effective treatments for combating the *Salmonella* pathogen.

5.1.2 *Salmonella* and Nitric Oxide

NO is an essential component of the host innate immune system response to pathogens such as *Salmonella*. It is produced by the iNOS enzymatic complex within macrophages following recognition of the PAMPs of invading micro-organisms (Umezawa *et al*, 1997). Mice which lack functional alleles of *iNOS* are considerably more susceptible to *Salmonella* infection compared to mice posessing operative *iNOS* alleles. Whilst control mice survive *Salmonalla* infection, *iNOS*-deficient mice subsequently perish from lethal

overgrowth of *S.* Typhimurium in the organs (Mastroeni *et al,* 2000). Other studies have correspondingly shown that prevention of NO synthesis in murine models results in significant increase of *S.* Typhimurium proliferation during infection (Umezawa *et al*, 1997; MacFarlane *et al*, 1999). MacFarlane *et al*, 1999 demonstrated that when NO is blocked *in vivo,* mice suffered increased mortaility due to an inability to clear *Salmonella* from the liver and spleen and displayed persistant bacteremia compared to untreated control mice. Such results substantiate the importance of NO in the control of *Salmonella* infection.

As well as NO, oxidative stress from reactive oxygen species (ROS) function as a defence to *Salmonella* infection. ROS such as the superoxide anion ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) are produced in macrophages in a respiratory burst performed by the nicotinamine adenine dinucleotide phosphate reduced (NADPH) oxidase as a result of the transient consumption of oxygen (Torres *et al*, 2006). Immune cells such as macrophages which posses the NADPH oxidase enzyme can utilize oxidative stress against invading pathogens. Highly reactive oxygen species, like RNS, are capable of damaging the DNA, RNA, proteins and lipids of *Salmonella* (Cabiscol *et al*, 1999). When $O_2^{\cdot -}$ reacts with NO the molecule produced is peroxynitrite ($ONOO^-$), an extremely powerful oxidant extensively damaging to DNA and other bacterial components. The bacteriostatic and bacteriocidal effects of such ROS and RNS make them a considerable impediment to *Salmonella* survival. As such, it is unsurprisng that *Salmonella* has developed mechanisms by which to withstand both of these stressors.

5.1.2.1 Nitric Oxide detoxfication

The role of the metalloenzyme flavohaemoglobin (HmpA), encoded by *hmpA* in *Salmonella*, is well characterised in NO detoxification. NO is oxidized to nitrate by HmpA in aerobic conditions or reduced to nitrous oxide by HmpA in anaerobic conditions. The enzyme is activated in the presence of NO, with its expression demonstrated to be highly induced in *S.* Typhimurium inside macrophages (Prior *et al,* 2009). The relevance of HmpA in NO detoxification is supported by the attenuation of *S.* Typhimurium *hmpA* mutants inside macrophages and by studies showing HmpA-deficient mice perish from *S.* Typhimurium infection before they can produce a nitrosative burst. As such, flavohaemoglobin has an evident role in *Salmonella* survival where it appears to be the prevailing enzyme in NO detoxification. However, other genes have been identified which may share involvement in NO resistance and detoxification in *Salmonella*. The exploration of other such potential NO detoxification genes is a focus of this research.

This study is based on preliminary data indicating that a number of largely uncharacterized *S.* Typhimurium genes were upregulated in response to nitrosative stress. The aim of this research was therefore the investigation and characterisation of these genes and their potential involvement in *S.* Typhimurium nitrosative and oxidative stress resistance.

5.2 Characterisation of possible NO-detoxifing genes

5.2.1 Mutants: Construction and Growth Curves

Mutant strains of the genes of interest were constructed using phage λ red recombination as has been previously described. Resulting bacterial colonies were checked for the presence of the internal pKD3 or pKD4 antibiotic resistance casette by PCR amplification with both external and internal verification primers, therefore confirming successful mutagenic strains. This process yielded twelve distinct mutants, including single, double, triple and quadruple mutant strains.

Before sensitivity to either oxidative or nitrosative stressors could be examined it was vital to determine whether any of the mutant strains displayed growth defects as a result of their mutation, as any such deficiencies would unfairly distort later data. All mutant strains were therefore grown alongside the isogenic parental strain SL1344 (WT) in nutrient rich LB media for a period of 24 hours with growth measured hourly up to the 8th hour by the OD_{600} of a 1mL aliquot. Encouragingly, all strains showed growth rates akin to that of the WT strain and reached similar final optical densities.

5.2.2 Mutants: Sensitivity towards NO

Once it had been established that all mutant strains displayed normal growth patterns and rates, the strains were exposed to nitrosative stress in the form of detaNONOate in order to examine whether they showed increased sensitivty to NO in comparison to the *S.* Typhimurium WT. Strains were subjected to the NO donor Deta NONOate at physiologically relevant levels

under both anaerobic and aerobic conditons. Deta NONOate spontaneously dissociates in a pH-dependent manner to release 2 moles of NO per mole of parent compound and has been used previously by the Rowley lab. The examination of mutant sensitivty to NO under both aerobic and anaerobic conditons allowed determination of whether mutated genes showed varied involvement in NO detoxification depending on oxygen availability, as in the case of the metalloenzymes *hmpA*, *norV* and *nrfA.*

Under aerobic conditions single mutants ∆*STM1250,* ∆*STM4452,* ∆*STM1586,* ∆*STM3362* and ∆*ycfR* do not appear to be more sensitive to NO than WT SL1344. However the considerably hindered growth of the ∆*STM1808-yeaR-tehB-HmpA* quadruple mutant, priorly shown to be highly susceptible to NO killing, demonstrated that the assay was functioning. When the assay was performed again under anaerobic conditons, mutants similarly did not display increased sensitivity to NO compared to the WT strain. In fact, mutant strains seemed to show improved survival over the SL1344 WT strain. Such results suggest that these single genes are not involved in NO detoxification as their absence does not hinder bacterial growth in the presence of NO. This includes ∆*ycfR* which has previously shown to be a resistance gene in both *E. coli* and *Salmonella* against oxidative and other stresses and was therefore most expected to show increased NO senstivity (Eletsky *et al*, 2014).

Aerobic and anaerobic NO sensitivity assays were also carried out with ∆*STM1250*, the double mutants ∆*ibpA-ibpB* and ∆*STM1250-STM1251,* and the quadruple mutant ∆*ibpA-ibpB-STM1250-STM1251.* ∆*STM1808-yeaR-*

tehB-HmpA was again used to demonstrate the functionality of the assay. In both aerobic and anaerobic conditions all multiple mutants displayed increased sensitivity to NO in comparison to the WT strain. Under anaerobic conditons both ∆*ibpA-ibpB* and ∆*STM1250-STM1251* show similar sensitivity to NO, surprisingly greater than that of the quadruple mutant. However under anaerobic conditons, ∆*ibpA-ibpB* displayed the greatest sensitivity out of all mutants, followed by ∆*ibpA-ibpB-STM1250-STM1251* and with ∆*STM1250-STM1251* the least effected. Such results suggest that *STM1250, STM1251* and their homologues *ibpA* and *ibpB* may have a role in *Salmonella* survival of nitrosative stress, but that *STM1250* and *STM1251* may be less vital and active more so under aerobic conditions. This is supported by the absence of *STM1250* and *STM1251* in similar microorganisms such as *E. coli*, which are able to withstand NO killing without either of these genes. As previously suggested, it is possible that the double mutant ∆*ibpA-ibpB* is more sensitive to NO than ∆*ibpA-ibpB-STM1250-STM1251* due to some compensatory mechanism acting in the latter, which is active due to both *ibpA*, *ibpB* and its homologues, *STM1250* and *STM1251*, being knocked-out. It is possible the bacterium recognizes the absence of both homologue pairs and the resulting compensation improves bacterial resistance to NO above that of the ∆*ibpA-ibpB* mutant. IbpA and IbpB are both heat shock proteins upregulated under stressors such as heat, oxidative stress and acid where they act as chaperones for damaged proteins. IbpA and IbpB interact in an as yet undefined manner and localise to the denatured protein, utimately facilitating its refolding. It has been demonstrated that an absence of IbpA and IbpB results in increased aggregation of proteins in *E. coli* cells under heat stress

(Matuszewska *et al*, 2008). Whilst there is no evidence in the current literature of their action in relation to nitrosative stress, the role of IbpA and IbpB in general stress response, their up-regulation in micrroaray data together with results of this study make it a distinct posibility that they aid in *Salmonella* resistance to NO killing.

As results from both sensitivity and susceptibility assays showed ∆*ibpA-ibpB-STM1250-STM1251* to be more greatly effected by NO than the WT strain the next step was to determine the contribution of the homologous pairs composing this quadruple mutant to NO resistance. To further investigate the individual NO sensitivity of ∆*ibpA-ibpB,* ∆*STM1250-STM1251 and* ∆*ibpA-ibpB-STM1250-STM1251* a technique known as an oxidative stress susceptibility assay described in Testerman *et al*, 2002 was adapted for nitrosative stress. Deta NONOate was again employed as an NO donor and was added to strain cultures in the respective quantites of 0mM and 7.5mM. Results from this assay further complement those achieved by NO sensitivity assays, with ∆*ibpA-ibpB* the most susceptible to NO killing and ∆*STM1250-STM1251* the least effected by NO. This supports the before mentioned theory that while STM1250 and STM1251 are involved in NO stress resistance, IbpA and IbpB play the more significant role of the two homologous pairs in NO detoxification.

5.2.3 Mutants: Sensitivity to H_2O_2

As ROS are also an important part of the host innate immune response to *Salmonella* infection, the multiple mutants which showed sensitivity to NO

were then exposed to hydrogen peroxide as a source of oxidative stress. Two distinct assays were utilized to determine whether multiple mutants were more sensitive to oxidative stress than the WT; sensitivity assays, as as had been previously performed for nitrosative stress, and oxidative stress susceptibility assays as per Testerman *et al*.

Sensitivity assays were performed with ∆*STM1250,* ∆*ibpA-ibpB,* ∆*STM1250-STM1251* and ∆*ibpA-ibpB-STM1250-STM1251* to determine sensitivity to oxidative stress, provided in the form of 3mM hydrogen peroxide. Results suggest that mutant strains are more sensitive to oxidative stress than the WT strain, with ∆*ibpA-ibpB* showing the greatest sensitivity. This reflects results achieved for nitrosative stress sensitivity assays, where growth of ∆*ibpA-ibpB* was most affected. Similarly, this result is not unpresedented as IbpA and IbpB have both previously been shown to have a role in resistance to oxidative stress in *E. coli*. Kitagawa *et al*, constructed IbpA, IbpB and IbpAB-overexpressing strains which were found to be more resistant to both oxidative stress and heat stress than the *E. coli* WT strain. *E. coli* strains deficient in *ibpA* and *ibpB* also appear to be more sensitive to superoxide radicals generated by tellurite (Perez *et al*, 2007). Similarly *Matsuszewka* et al, demonstrated that IbpA and IbpB were involved in resistance to copper induced oxidative stress in *E. coli* cells. Most recently it was observed that IbpA and IbpB are upregulated in non-pathogenic *E. coli* in an ROS-dependent manner in macrophages, which then serves to protect the bacterium from oxidative stress (Goeser *et al*, 2015). Such evidence supports the findings of this study. It is therefore likely that both *ibpA* and *ibpB* are

somehow involved in both nitrosative and oxidative stress tolerance in *S.* Typhimurium.

Oxidative stress susceptibility assays, performed with the ∆*ibpA-ibpB-STM1250-STM1251* and ∆*STM3362-ycfR-ybiJ* triple mutant in comparison to the WT strain, showed ∆*ibpA-ibpB-STM1250-STM1251* to be the most susceptible to oxidative stress. ∆*STM3362-ycfR-ybiJ* also showed increased sensitivity to H_2O_2 in comparison with the WT, though to a lesser degree than ∆*ibpA-ibpB-STM1250-STM1251*. As such it is plausible that the individual genes *STM3362, ycfR* and *ybiJ* may be involved in oxidative stress resistance in *Salmonella*. All share a domain of unknown function, DUF1471, which has been found to be present largely in proteins involved in the stress responses of *Salmonella* and *E. coli* (Eletsky *et al*, 2014). Little is known about this domain or the majority of proteins containing it apart from this, but results here suggest that genes such as *STM3362, ycfR* and *ybiJ* may indeed have minor roles in oxidative stress tolerence.

5.3 Mutants: Conclusions

Therefore, whilst a number of genes were shown to be up-regulated in the initial microarray, results of both sensitivity and susceptibility assays failed to show increased NO sensitivity in ∆*STM1250,* ∆*STM4552,* ∆*STM1586,* ∆*STM3362* or ∆*ycfR.* However, the triple mutant ∆*STM3362-ycfR-ybiJ* demonstrated susceptibility to oxidative stress and multiple mutants ∆*STM1250-STM1251,* ∆*ibpA-ibpB* and ∆*ibpA-ibpB-STM1250-STM1251* all

demonstrated increased susceptibility to both NO and H_2O_2, suggesting a role for these genes in *Salmonella* oxidative and nitrosative stress resistance.

5.4 HilA and the regulation of Nap nitrate reductase

5.4.1 Context

As a faculative anaerobe *Salmonella* is capable of switching to nitrate respiration, allowing it to survive in oxygen-limited conditions. This is known as the dissimilatory from nitrate to ammonium, or DNRA pathway, where *Salmonella* produces energy for respiration by reduction of nitrate to nitrite via nitrate reductase enzymes. NO is a by-product of this reduction of nitrate, which itself undergoes truncated dentrification to nitrous oxide (N_2O) (Sparacino-Watkins *et al,* 2014).

Salmonella synthesises two distinct enzymes which reduce nitrate to nitrite; a cytoplasmic membrane bound enzyme known as NarG, and the periplasmic nitrate reductase, NapA. In the cytoplasm, NarGHI reduces nitrate to nitrite, and NirB further reduces this nitrite to ammonium, whilst in the periplasm, NapA reduces nitrate to nitrite and a periplasmic cytochrome c nitrite reductase (NrfA) performs the conversion to ammonium. It is thought that under nitrate-limited conditions Nap is unregulated and performs as the main nitrate reductase, as it has a higher affinity for nitrate, whilst under nitrate-rich conditions, Nar is utilised (Rowley *et al*, 2012).

Whilst little is known about the regulation of Nap, preliminary data revealed that the expression of five genes of the *napF* operon overlapped with those of

SPI-1 in hierarchal cluster analysis. SPI-1 encodes the type three secretion system (TTSS) of *Salmonella* and most associated effector proteins responsible for the bacterium's invasion capabilities. SPI-1 itself is controlled by the master regulator and transcriptional activator, Hyperinvasion Locus Protein A, otherwise known as HilA. It was therefore proposed that HilA might possibly also regulate the *napF* operon, and furthermore, that clustering of SPI-1 and Nap genes might indicate a potential role for *Nap* in *Salmonella* invasion. This study therefore aimed to investigate whether HilA is a regulator of nitrate reductase, NapA.

5.4.2 HilA as a possible negative regulator of Nap

To determine whether HilA is a regulator of the *napF* operon, *S.* Typhimurium strains containing a *nap-lacZ* fusion were utilized in both aerobic and anaerobic β-galactosidase assays. Different *in vivo* conditions were simulated by use of varied media, including rich LB media and high-salt LBS, the latter of which is known to induce the expression of SPI-1 and therefore HilA. Should HilA act as a positive regulator of NapA, the enzyme's expression should be highest under SPI-1 inducing conditions.

Whilst experiments in LB and LBS were performed aerobically, cultures grown in M9 minimal media were done so anaerobically. Both low nitrate, high glycerol M9 (N^-/G^+) and high nitrate, low glycerol M9 (N^+/G^-) media was used, with the former being theoretically the ideal conditions for the induction of NapA expression.

However, assay results from all media conditions suggest that HilA is not a positive regulator of the *napF* operon, as the fusion strain possessing *hilA* did not show increased NapA expression in comparison to the *hilA* deletion strain under any media conditions. Conversely, in all conditions, particularly that of aerobic LB cultures, NapF expression is slightly higher in the *hilA* deletion mutant. This unexpected result seems to suggest an inhibitory role for HilA in regards to NapF. Although no current literature addresses the possibility of HilA as a transcriptional repressor, results achieved here reflect those achieved previously by Prior, 2011, in which slightly higher activity of NapF in the *hilA* deletion background was again observed. It is therefore the conclusion of this study that HilA may exert a negative regulatory effect on the Nap nitrate reductase of *Salmonella*.

5.4 Future work and concluding remarks

STM3362, ycfR and *ybiJ* were not individually examined for sensitivity to oxidative stress, so their individual contribution to the oxidative stress tolerance of *S.* Typhimurium is unknown. In light of results demonstrating Δ*STM3362-ycfR-ybiJ* susceptibility to oxidative stress, H_2O_2 sensitivity assays should be performed comparing Δ*STM3362,* Δ*ycfR*, Δ*ybiJ* Δ*STM3362-ycfR-ybiJ* oxidative stress sensitivity to that of the WT. Similarly, nitrosative stress sensitivity assays should be performed in the same manner, as neither Δ*ybiJ* nor Δ*STM3362-ycfR-ybiJ* were examined for sensitivity to NO within this study. This would allow determination of whether Δ*STM3362-ycfR-ybiJ* displays susceptibility to nitrosative stress as well as oxidative stress.

Results of this study demonstrate that ∆*ibpA-ibpB* is considerably more sensitive to NO than ∆*STM1250-STM1251* under aerobic conditons, however both mutants display similar NO sensitivity in anaerobic conditons. This may indicate that *STM1250* and *STM1251* contribute more to NO resistance under anoxic conditons than they do in aerobic environments or that *ibpA* and *ibpB* are more protective against NO killing under aerobic conditions than anaerobic ones. Based on strain survival across the two assays the latter appears to be the more plausible possibility, however further NO sensitivity assays would be required to determine this.

Following further examination of the sensitivity of mutant strains, future research should focus on cell culture. Gentamicin sensitivity assays in both resting and IFN-γ activated macrophages should be performed to assess the ability of mutant strains to infect and survive in macrophages in comparison with the WT strain. Of particular interest will be the double mutant Δ*ibpA-ibpB* and the quadruple mutant, Δ*ibpA-ibpB-STM1250-STM1251*, which both displayed oxidative and nitrosative sensitivity.

A further worthwhile venture would be the transformation of GFP-plasmids into mutant strains, allowing for observation of intracellular trafficking and survival of both mutants and WT inside macrophages, both in the absence and presence of NO-inhibitors. If mutants display particular sensitivity to NO-killing the introduction of an NO-inhibitor such as L-NAME or L-NIL would be expected to rescue mutant strain expression. The interaction of mutant strains and NO could also potentially be observed by use of a flourescence based

NO-sensor to investigate the interaction of NO and mutants within macrophages.

As results of this study suggest that HilA may negatively regulate Nap future work should seek to confirm this by use of *hilA* mutants, as well as ChIP-on-chip and ESMA.

To conclude, this study suggests roles for various genes, such as *ibpA* and *ibpB* in NO detoxification and survival of oxidative stress in *S.* Typhimurium. However further investigation in live cell cultures is needed to fully elucidate the role of such genes and their importance in resistance to nitrosative stress.

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