Mechanisms of anaerobic nitric oxide detoxification by *Salmonella enterica* serovar Typhimurium

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Abstract

Salmonella is the cause of millions of food- and water-borne infections worldwide. Systemic infection and gastroenteritis are the main diseases and often prove fatal to immunocompromised patients.

Key to *Salmonella'*s pathogenicity is the survival of several components of the innate immune system encountered during infection. Reactive oxygen and nitrogen species (ROS and RNS) are an integral part of this antibacterial defence of the immune system. Exposure to ROS and RNS occurs within phagocytic immune cells such as macrophages, where such generation of radicals is used to combat pathogens. NO is a radical belonging to the group of RNS that damages bacterial DNA and proteins. Detoxification of NO is essential during infection to allow *Salmonella* to survive and replicate within macrophages. Three enzymes are currently known to help *Salmonella* to detoxify NO, but their deletion, however, does not eliminate *Salmonella*'s survival. Therefore, it is predicted that further mechanisms for NO detoxification exist.

In this study, the core NO regulon has been identified: Expression of nine genes is significantly increased during endogenous and exogenous NO exposure of *S*. Typhimurium. Their functions range from carbon starvation, cytochrome oxidation, iron-sulphur repair and NO reduction to putative proteins with unknown function, some of which contain domains for tellurite resistance. Single and combination deletion strains have shown that these genes are important to decrease anaerobic NO sensitivity of *S*. Typhimurium and for intracellular survival in murine macrophages. Furthermore, we have shown for the first time that the core NO regulon also provides protection against tellurite. Tellurite is toxic and requires detoxification when encountered. Reducing tellurite to yield the elemental tellurium results in the release of ROS, which then need to be detoxified further. Deletion strains sensitive to tellurite have also shown increased sensitivity to NO. Concurrently, tellurite resistance genes also facilitate the defence against NO.

Table of contents

List of Figures

List of Tables

Abbreviations

Abbreviation:	Explanation:
[Fe-S]	iron-sulphur
A	absorption
ACSSuT	Ampicillin, Chloramphenicol, Streptomycin, Sulfonamide, Tetracycline
Amp	Ampicillin
BMDM	bone-marrow derived macrophages
bp	base pairs
Cat	Chloramphenicol
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
cfu	colony-forming units
$CHCl_3$	chloroform
CRP	cAMP-regulator protein
C_t	threshold cycle value
Cu,ZnSOD	Cu,Zn superoxide dismutase
dH_2O	distilled water
DMEM	Dulbecco's modified Eagle medium
DTT	dithiothreitol
DUF	domain of unknown function
EDTA	ethylenediaminetetra-acetic acid
eNOS	endothelial nitric oxide synthase
EPR	electron paramagnetic resonance
FCS	foetal calf serum
FMN	flavin mononucleotide, riboflavin-5'-phosphate
FNR	fumarate and nitrate reductase regulator
FRT	FLP recognition targets
Fur	ferric uptake regulator
F/S	filter sterilized
GC	gas chromatography
GSH	glutathione
GSNO	*S*-nitrosoglutathione
H_2O_2	hydrogen peroxide
Hcy	homocysteine
HGT	horizontal gene transfer
HPLC	high-performance liquid chromatography
IFN-γ	interferon gamma
IgG	immunoglobin G
IL	interleukin
iNOS	inducible nitric oxide synthase

Abbreviation:	Explanation:
iNTS	invasive non-typhoidal *Salmonella*
K_2TeO_3	potassium tellurite
Kan	Kanamycin
kb	kilo base pairs
LB	Luria-Bertani
LPS	lipopolysaccharides
M cells	microfold cells
MDR	multi-drug resistant
MES	2-(*N*-morpholino)ethanesulfonic acid
MGM	minimal glucose medium
MIC	minimal inhibitory concentration
MOI	multiplicity of infection
N_2O	nitrous oxide
NH_4	ammonia
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NO^-	nitroxyl
NO_2^-	nitrite
NO_3^-	nitrate
NOS	nitric oxide synthase
NsrR	nitric oxide-sensitive repressor
NTS	non-typhoidal *Salmonella*
$O_2^{\cdot\cdot}$	superoxide anion
OD	optical density
OH^-	hydroxyl radical
ONPG	*o*-nitrophenyl-β-D-galactopyranoside
OPS	O polysaccharides
PAMP	pathogen-associated molecular pattern
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
Phox	NADPH oxidase / phagocytic oxidase
PMN	polymorphonuclear leukocytes
ppm	parts-per-million
qRT-PCR	quantitative real-time reverse transcription PCR
rEPA	*Pseudomonas aeruginosa* recombinant exoprotein A
RNI	reactive nitrogen intermediates
RNS	reactive nitrogen species
ROS	reactive oxygen species
rpm	revolutions per minute
RT-PCR	reverse transcription polymerase chain reaction
SAM	*S*-adenosyl-L-methionine

Abbreviation:	Explanation:
SCV	*Salmonella*-containing vacuole
SNAP	*S*-nitroso-*N*-acetyl penicillamine
Sper/NO	Spermine-NONOate
SPI	*Salmonella*-pathogenicity island
SSPE buffer	saline-sodium phosphate-EDTA buffer
st. dev.	standard deviation
TeO_3^{2-}	tellurite
TeO_4^{2-}	tellurate
Tet	Tetracycline
TNF-α	tumour necrosis factor alpha
TraDIS	transposon directed insertion site sequencing
Tris	tris(hydroxymethyl)-aminomethane
TTSS	type three secretion system
Vi	virulence capsular polysaccharide of *S*. Typhi
(v/v)	volume per volume in relation to 100 mL
WHO	World Health Organisation
(w/v)	weight per volume in relation to 100 mL

1 Introduction

1.1 *Salmonella*

1.1.1 Nomenclature

The bacterium *Salmonella* belongs to the class of γ-proteobacteria and is a member of the *Enterobacteriaceae* family (Le Minor & Popoff, 1987). There are only two *Salmonella* species, namely *S. bongori* and *S. enterica*, but the genus of *S. enterica* comprises a vast list of more than 2500 different serovars (Andrews-Polymenis et al, 2010; Popoff et al, 2003). Most commonly associated with infection in humans are the serovars *Salmonella enterica* ssp. *enterica* serovar Typhi (*S*. Typhi), *Salmonella enterica* ssp. *enterica* serovar Paratyphi (*S*. Paratyphi), *Salmonella enterica* ssp. *enterica* serovar Typhimurium (*S*. Typhimurium) and *Salmonella enterica* ssp. *enterica* serovar Enteritidis (*S*. Enteritidis). Serovars are often differentiated by adaption or restriction to certain hosts, e.g. to pigs (*S*. Dublin) and poultry (*S*. Gallinarum). For humans, host-restricted serovars include *S*. Typhi, *S*. Paratyphi A and *S*. Paratyphi C. Most serovars, however, are not host-restricted as they infect a broad range of animals, often causing self-limiting infections (Rabsch et al, 2002). The serovar *S*. Typhimurium has been isolated from humans, cattle pigs and poultry where it either causes gastroenteritis or results in asymptomatic carrying. However, certain serotype variants of *S*. Typhimurium have been shown to cause paratyphoid in pigeons (Milnes et al, 2008; Rabsch et al, 2002; Snow et al, 2007). Hence it has to be noted that *Salmonella* is the causative agent for infection of a range of animals and leads to enteric fever.

1.1.2 Epidemiology of Salmonellosis

In humans *Salmonella* infection results in two major disease outcomes, gastroenteritis and enteric fever. Enteric fever is a systemic infection caused by *S*. Typhi then referred to as typhoid fever, and by *S*. Paratyphi, leading to paratyphoid fever. As these serovars are host-restricted, humans serve as reservoirs with an infection spreading via the faecal-oral route (Okoro et al, 2012). The genome of *S*. Typhi is approximately 10% different to that of the non-typhoidal *Salmonella* (NTS) strain *S.* Typhimurium, mostly due to the accumulation of pseudogenes in *S*. Typhi (Young et al, 2002), but also due to

the acquisition of pathogenicity islands (SPI) by horizontal gene transfer (HGT) such as SPI-VII which encodes for the Vi capsule. The *S*. Typhi genome also contains genes for a type IV pilus, a putative type IV secretion system as well as a phage encoding for the *sopE* effector gene of SPI-1 (Deng et al, 2003; McClelland et al, 2001; Parkhill et al, 2001). A genome comparison between *S*. Typhi and *S*. Typhimurium also revealed the inactivation of 5% of genes in *S*. Typhi, including genes important for intestinal persistence (*ratB* and *shdA*) and other genes from several SPIs (Baker & Dougan, 2007). This provides an explanation to why *S*. Typhi infection differs from *S*. Typhimurium infection and e.g. is restricted to the human host and can infect other organs such as the gallbladder.

S. Typhimurium, unlike *S*. Typhi, is a non-human restricted serovar and infects a range of animals from chickens and other poultry, to mice and pigs (Milnes et al, 2008; Okoro et al, 2012). Infection of humans by NTS strains normally results in a self-limiting gastroenteritis, whereas infection of mice leads to typhoid-like symptoms and a high fatality rate, providing us with an excellent animal model for Typhoid fever (Prior et al, 2009).

The highest risk of Typhoid infection is in south-central and south-east Asia, with medium risk for African, other Asian, Latin American, Caribbean countries and Oceania excluding Australia and New Zealand (Crump et al, 2004). In the year 2000, an estimated total of more than 26 million cases of enteric fever caused by *S*. Typhi and *S*. Paratyphi occurred, leading to 200,000 - 500,000 deaths each year (Crump et al, 2004; Everest et al, 2001). Although incidence rates have decreased in some countries, such as Vietnam, due to improved sanitation, in other countries such as Indonesia and Pakistan there has been little impact on the number of infections recorded (Andrews-Polymenis et al, 2010; Pang et al, 1998). This also coincides with a fatality rate of up to 7% in Pakistan, compared to an average of 0-5% in other countries (Pang et al, 1998).

According to the World Health Organisation (WHO) approximately 1.3 billion cases of gastroenteritis per annum are caused by *Salmonella* (Pang et al, 1995). Every year, more than ten thousand individuals in England and Wales are diagnosed with Salmonellosis of this type, making it the second most common cause of food poisoning in these regions (Health Protection Agency,

2009). *S*. Enteritidis and *S*. Typhimurium have been reported as the two most common serovars causing the infections in England and Wales. Prevalence of NTS cases is high in sub-Saharan Africa, but not limited to this geographical area (Kingsley et al, 2009). Approximately 3 million deaths are estimated to occur every year as a result of infection with NTS (Pang et al, 1995). Most of these deaths are linked to the immunocompromised population, suffering from Malaria or infection with HIV (Gilks et al, 1990; Gordon et al, 2002; Hohmann, 2001; Vugia et al, 1993). As the symptoms of *Salmonella*-caused gastroenteritis are similar to those caused by other food poisoning micro-organisms, along with the fact that the disease is usually self-limiting, make this an under-reported disease (Prior et al, 2009). The economic burden of this disease remains high; the cost for the National Health Service (NHS) for treatment of Salmonellosis has been estimated to lie between £1,000 to £1,200 per case in the UK (Santos et al, 2011). Laboratory tests of samples alone cost the NHS £6.5 million each year and in countries with weaker health infrastructures, cases are further underreported as health care is less available to the particularly vulnerable young children and the elderly (Parry et al, 2002; Prior et al, 2009).

1.2 Infection biology of *Salmonella*

1.2.1 Route of infection

Human infection with *Salmonella* ssp. occurs after the ingestion of bacteria from contaminated food or water. The bacteria reach the acidic environment of the stomach before being transported into the intestine. From here *Salmonella* causes infection of enterocytes, intestinal epithelial cells, and subsequent replication within macrophages takes place.

1.2.1.1 Sources of contamination

The consumption of meat, eggs or poultry from infected animals is the most common source of NTS infection in Europe (Adak et al, 2005). Cross-contamination of meat products post slaughter is an additional risk. Another source is the contamination of drinking water. Especially in countries where the supply of clean drinking water is not secured, contamination with human faecal

content can also lead to an infection (Parry et al, 2002; Tischler & McKinney, 2010).

If animal manure is used as a fertilizer during fruit and vegetable production, *Salmonella* readily adheres to the surface of the plant and even invades plant tissue (Gu et al, 2011; Klerks et al, 2007). An increasing number of outbreaks of foodborne illnesses caused by *S*. Typhimurium have been reported in the United States that originate from contaminated fruit and vegetables (Andrews-Polymenis et al, 2010). These are the result of using contaminated manure or irrigation water or the result of contamination during post harvest processes. *S. enterica* serovars Montevideo, Newport and Javiana have been most commonly linked with such outbreaks from produce (Shi et al, 2007). In comparison with serovars linked to meat-associated Salmonellosis, e.g. Enteritidis and Typhimurium, Montevideo and Newport have a higher attachment and infection rate of tomato and lettuce plants (Gu et al, 2011; Klerks et al, 2007; Shi et al, 2007; Zheng et al, 2013). It has determined that *Salmonella* can be found in ripe and unripe tomato fruits and that internalization presumably takes place via the stomata in the leaves. Infection of plants has a higher success rate when the blossom or leaves of the plants are exposed to the bacterial cells, in comparison with low infection rates via the soil-root interface (Klerks et al, 2007; Zheng et al, 2013). Low intracellular rates of *S*. Typhimurium have been measured in several studies and contamination of fruit products with this serovar is linked to food processing rather than contamination during crop growth. Investigation of a *S*. Newport mutant library has identified a number of genes including *agfBD* and *rpoS* that are particularly important during the attachment of *S*. Newport to alfalfa sprouts (Barak et al, 2005). The rdar morphotype is characterised by the formation of dry and rough colonies and plays an important role for environmental persistence (Gu et al, 2011). The transcription factor AgfD regulates the expression of *agfB* (fimbriae), *yihO* (O antigen capsule formation) and *bscA* (cellulose synthesis), which are key for the rdar morphotype (Barak et al, 2005; Gu et al, 2011). Mutations within *agfD* or its deletion results in significantly decreased attachment properties of the strains tested (Gu et al, 2011). It has been concluded that this morphotype is beneficial

for the survival on the surface of leaves, but does not provide any benefits once the bacteria are inside the leaves.

However, several food preparation techniques significantly reduce the number of bacteria in food products and these include thorough cooking of meat and vegetables and boiling of drinking water (Adak et al, 2005). This is of course more problematic with salad crops or other vegetables intended to be eaten raw; washing these products is not always sufficient to remove *Salmonella* when they are residing within plant cells. The increase in *Salmonella* infections associated with such products correlates with dietary advice on increasing fruit and vegetable consumption, and subsequent mass production of these products.

1.2.1.2 Gastric passage and gut commensals

After ingestion of contaminated food or water, the next step during infection is the transport of *Salmonella* into the stomach, where the low pH of the gastric juice provides an effective barrier for a range of pathogens. *Salmonella* uses the acid tolerance response (ATR) to counteract and adapt to this environment (Garcia-del Portillo et al, 1993). Via the regulators σ^S and Fur, the expression of more than fifty acid shock proteins, including the proton-translocating ATPase (Atp), is induced that protects *Salmonella* against extreme low pH values such as pH 3.0 to 4.0 (Foster, 1991; Gahan & Hill, 1999; Hall & Foster, 1996). The ferric uptake regulator Fur and σ^S have been identified to initiate the ATR in log phase cells, as a response to the increased level in organic acids. Additional regulators include the two-component systems PhoPQ and OmpR-EnvZ (Rychlik & Barrow, 2005). PhoPQ initiates the ATR when inorganic acids have lowered the pH. Protection of stationary phase cells is provided through the activation of the ATR by the OmpR-EnvZ sensory system.

After passage through the stomach, *Salmonella* reaches the small intestine. Here, the commensal gut flora reduces pathogenicity of intruding infectious bacteria by competing for available nutrients and adhesion receptors, but also through the production of antibacterial metabolites, such as bacteriocins and short chain fatty acids (Álvarez-Ordóñez et al, 2011). Goblet cells, located in the intestinal epithelium, produce a layer of mucus that protects the enterocytes. The inner layer of this mucus layer is dense and lacks microbiota, whereas the

outer layer is looser and houses commensal bacteria (Gill et al, 2011). The short chain fatty acid butyrate produced by commensals triggers an increase in mucus production and further increases the protection of enterocytes against pathogens. It has been shown that *Salmonella* requires chemotaxis and flagella to be able to penetrate the mucus layer (Stecher et al, 2004). During *Salmonella* infection, the levels of commensal bacteria decrease significantly due to the stimulation of the host inflammation response (Deatherage Kaiser et al, 2013). This allows increased proliferation of *S*. Typhimurium in the infected gut and increases the invasion rate. In addition, *Salmonella* stimulates the expression of RegIIIβ, a bactericidal compound, by host cells that reduces the growth of commensal bacteria (Stelter et al, 2011). Protection of *S*. Typhimurium against the bactericidal activity of RegIIIβ was shown to result from the presence of O-antigens in the bacterial cell wall.

Recognition of the cystic fibrosis transmembrane conductance regulator (CFTR) found in the membrane of intestinal mucosal cells has been associated with enhanced invasion of enterocytes by *S*. Typhi, but not *S*. Typhimurium (Lyczak, 2003; Pier et al, 1998). This is another indicator for differences between the course of infection of *S*. Typhi and *S*. Typhimurium that lead to two major disease types of enteric fever and gastroenteritis.

1.2.2 Gastroenteritis

S. Typhi triggers a systemic infection of the human body, whereas *S*. Typhimurium infections is stopped at the mesenteric lymph nodes, leading to a localised intestinal inflammation that manifests itself through diarrhoea (Young et al, 2002). *Salmonella*-specific mechanisms such as the type three secretion systems (TTSS) encoded on *Salmonella*-pathogenicity islands-1 (SPI-1) and -2 (SPI-2) are important during stages of infection of both serovars and are discussed here.

S. Typhimurium multiplies in the intestinal lumen and like *S*. Typhi also uses the gut filtering properties of microfold cells (M cells) to reach the basolateral side of the epithelial tissue of the intestine. In addition, it also invades enterocytes to avoid antibacterial enzymes (Figure 1).

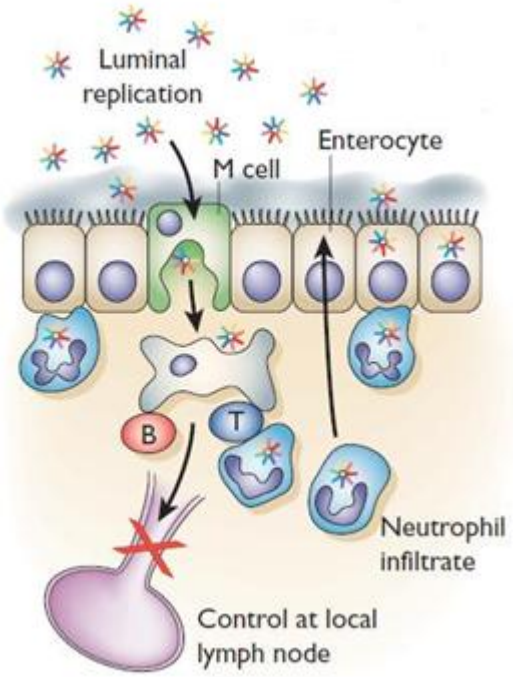

Figure 1: Intestinal entry of *S*. Typhimurium (Young et al, 2002)

S. Typhimurium replicates within the gut lumen and reaches the basolateral surface of the intestinal epithelial layer through passage of M cells or by the active invasion of enterocytes, where they are taken up by phagocytic cells such as neutrophils and the infection is controlled at the mesenteric lymph nodes. Infiltration of neutrophils into the intestinal lumen contributes to the inflammation of the tissue and leads to diarrhoea.

1.2.2.1 Role of SPI-1

Active invasion is made possible by the expression of a needle-like complex and subsequent secretion of so-called effector proteins from a set of genes belonging to SPI-1. This region on the *S*. Typhimurium genome comprises more than 30 genes (Collazo & Galán, 1997; Marcus et al, 2000; McGhie et al, 2009). Regulation of SPI-1 is complex and a range of environmental factors and transcriptional regulators have been identified, which influence SPI-1 expression (Figure 2).

One regulatory trigger is the change in hydrogen ions (pH) that occurs during the passage of bacteria from the stomach to the small intestine. This change in proton concentration is sensed by PhoP-PhoQ, Fur and OmpR-EnvZ (López-Garrido & Casadesús, 2012; Rychlik & Barrow, 2005). These regulators modify the levels of the transcriptional regulators HilA, HilC, HilD and InvF that results in the expression of SPI-1 genes. Other environmental signals for SPI-1 activation include low oxygen concentrations and changes in nutrient levels (Darwin & Miller, 1999; Marcus et al, 2000).

The SPI-1 proteins fulfil a range of functions. Some are structural components, forming a needle-like complex of inner and outer membrane proteins that form a hollow tube and span across both the bacterial and eukaryotic membranes (Kubori et al, 1998). Another function is fulfilled by chaperones such as SicA that ensure correct folding of the proteins such as SipB and SipC (Tucker & Galán, 2000). Some components are effector proteins that are translocated from the bacteria into the eukaryotic host cell. These effector proteins, such as SopB, SipA and SipC, interact with the actin component of the host cell cytoskeleton (Finlay et al, 1991; Haraga et al, 2008). The subsequent rearrangement of actin induces the ruffling of the eukaryotic cell membrane and results in the uptake of the bacterial cell into the host cell in a vesicular compartment (Marcus et al, 2000; Stevens et al, 2009). Once membrane ruffling has led to the intracellular uptake of bacteria, the *Salmonella* protein tyrosine phosphatase SptP reverses the actin rearrangements caused by SopB (Fu & Galán, 1998; Fu & Galán, 1999; Humphreys et al, 2009; Patel & Galán, 2006). SptP mimics GTPase-activating proteins and hence deactivates the GTPases Cdc42 and Rac that are responsible for the actin modulation.

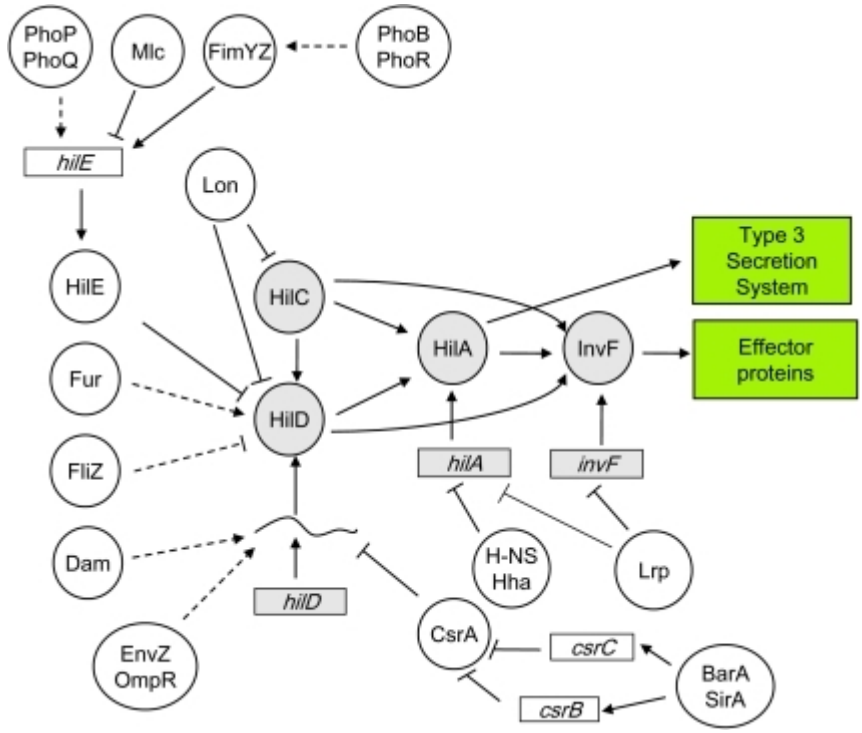

Figure 2: SPI-1 regulation (López-Garrido & Casadesús, 2012)

Regulatory input determining the levels of the SPI-1 regulators HilA, HilC, HilD and InvF (grey). Dashed lines indicate indirect, solid lines indicate direct regulation of genes (rectangular boxes) or proteins (circles), where blunt ends symbolize repression and arrows activation.

Therefore, *Salmonella* induces the rearrangement of the actin cytoskeleton of the host cell for invasion, but also reverses the effects post invasion. Other effectors (SopE, SopE2 and SopB) trigger the release of the signaling molecule interleukin-8 (IL-8) via the interference with MAPK pathways (Darwin & Miller, 1999; Haraga et al, 2008; Young et al, 2002). In turn, IL-8 stimulates the migration of polymorphonuclear leukocytes (PMN), white blood cells, into the lumen, resulting in intestinal inflammation and diarrhoea (Galyov et al, 1997). Such migration of PMN is not observed for *S*. Typhi or *S*. Paratyphi infections and hence their primary infection does not cause inflammation of the small intestine.

Once neutrophils, one type of white blood cells, have been recruited in response to a *S*. Typhimurium infection, they release prostaglandins which increase the activity of adenylate cyclases in enterocytes (Darwin & Miller, 1999). This enzyme then no longer regulates the absorption of sodium ions, but increasingly leads to the secretion of chloride ions into the intestinal lumen. This results in diarrhoea that cannot be easily distinguished from the symptoms caused by other enteric pathogens.

Furthermore, it has been shown that the SPI-1 effector SipB causes the formation of multi-membrane structures within the host cell which disrupt mitochondria (Hernandez et al, 2003). This induces autophagy leading to cell death and high cell toxicity of *S*. Typhimurium invading epithelial cells that has been reported in the literature (Hautefort et al, 2008; Hernandez et al, 2003; Jones et al, 1994).

Deletions of SPI-1 have highlighted that SPI-1 plays a role during oral infection, but is not required for the systemic phase of infection (Galán & Curtiss, 1989a; Jones & Falkow, 1994; Jones et al, 1994; Marcus et al, 2000).

1.2.2.2 Role of SPI-2

The invasion of epithelial and phagocytic cells results in *Salmonella* being present intracellularly within a vesicle or spacious phagosome (Haraga et al, 2008; Stevens et al, 2009). Within minutes, the vacuole shrinks and gives rise to the *Salmonella*-containing vacuole (SCV). Once *Salmonella* has invaded host cells, the transcription of a second SPI is induced (Cirillo et al, 1998; Hautefort

et al, 2008). SPI-2, like SPI-1, also encodes for a needle-like complex and effector proteins. Similarly, the pore structure allows translocation of effectors from the SCV into the host cell cytosol (Figueira & Holden, 2012). The role of SPI-2 includes four tasks (Figure 3). Firstly, it allows the acquisition of proteins from the cytosol of the host cell. Secondly, it allows the movement of the SCV towards the nucleus and the Golgi apparatus.

Thirdly, it allows bacterial multiplication within the SCV. Fourthly, it has been shown that SPI-2 can prevent the fusion of the inducible nitric oxide synthase (iNOS) with the SCV, thus reducing the exposure of *Salmonella* to RNS (Chakravortty et al, 2002). A more acidic pH and the low nutrient composition of phosphate and magnesium in the SCV are inducing conditions for SPI-2 expression and are sensed by the two-component systems SsrAB and OmpR-EnvZ (Cirillo et al, 1998; Eriksson et al, 2003; Feng et al, 2004; Figueira & Holden, 2012; Garmendia et al, 2003; Lee et al, 2000).

The identification of SPI-2 effectors has been more difficult than identifying SPI-1 effectors as they are scattered across the genome, outside of the pathogenicity island. Similar to SPI-1, SPI-2 also contains effectors that modify the host cytoskeleton. In particular the effector proteins SteC, SspH2 and SrfH have been shown to polymerise actin, to assemble an actin meshwork and to co-localise to the meshwork, respectively (Figueira & Holden, 2012). The effector proteins SseF and SseG recruit the cell motor protein dynein to the SCV and in combination with actin polymerisation, the SCV moves closer to the nucleus and to the Golgi apparatus. The reason for the benefits of a SCV closely associated with the microtubule organizing centre and the Golgi apparatus remains unknown (Ramsden et al, 2007).

Other effectors function mainly in the maintenance of the SCV membrane integrity (SifA, PipB2, SseJ, SopD2), but also modify signalling processes within the host cell and hence modify the immune signalling of cells (SpvC, SspH1). The environment within the SCV allows bacterial replication and it has been proposed that the formation of *Salmonella*-induced filaments (Sif) by the SPI-2 effector SifA might allow for the expansion of the SCV (Haraga et al, 2008).

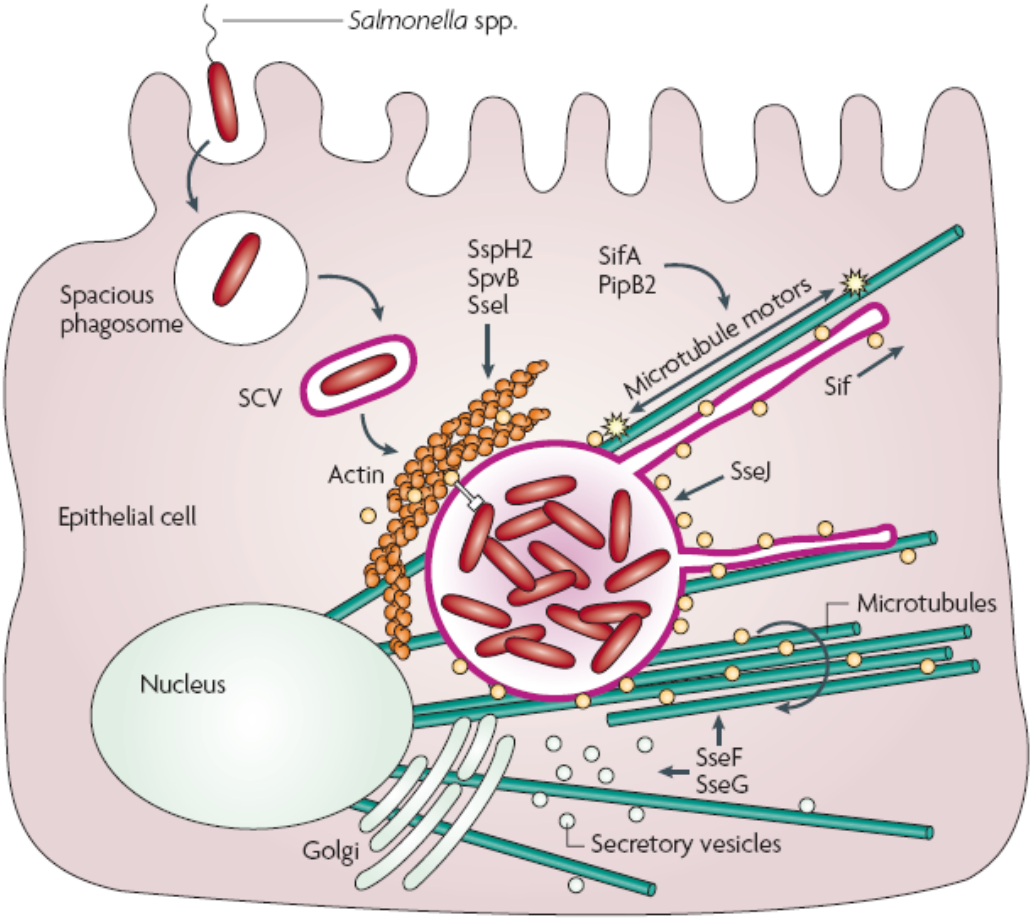

Figure 3: Entry of *Salmonella* into macrophages & survival within the *Salmonella*-containing vacuole (Haraga et al, 2008)

Salmonella cells are taken up into an intracellular compartment of macrophages, the spacious phagosome. This structure shrinks and forms the SCV. Several genes clustered on SPI-2 allow the acquisition of proteins from the cytosol of the macrophage, enable multiplication with the SCV and through movement of the SCV along microtubules, allow the close association of SCV to the Golgi apparatus and the nucleus. The fusion of the lysosome with the SCV acidifies the environment and exposes the cells to antimicrobial peptides and the toxicity of reactive oxygen and nitrogen species. SPI-2 effectors allow *Salmonella* spp. to avoid these bactericidal effects and ensure the successful survival and the further multiplication of the bacteria within the SCV.

Similarly to SPI-1, SPI-2 also has the ability to induce apoptosis of host cells, possibly by the activity of the effector SlrP as well as through the activation of caspase-1 activity (Figueira & Holden, 2012; Monack et al, 2001). Apoptosis of for example macrophages provides a way of escaping a further immune system attack once the macrophages reach the mesenteric lymph nodes. Transport of *Salmonella*-containing phagocytes to the mesenteric lymph nodes allows the presentation of antigens to B and T cells, hence stimulating the adaptive immune system and the production of antibodies.

Further protection for *Salmonella* is provided by partially preventing the fusion of lysosomes with the SCV, avoiding antimicrobial enzymes (Uchiya et al, 1999). The assembly of NAPDH oxidase is also disrupted, decreasing the exposure to reactive oxygen species (ROS) as the enzyme activity levels are low (Gallois et al, 2001; Vazquez-Torres & Fang, 2001b). Furthermore, the fusion of vesicles containing iNOS is furthermore inhibited, reducing the exposure to nitric oxide (NO) and other reactive nitrogen species (RNS) (Chakravortty et al, 2002). *Salmonella* has enough defence mechanisms to detoxify the antibacterial radicals encountered within the SCV and survives in this environment. These detoxification mechanisms are described in more detail in section 1.5.

The uptake of pathogens into macrophages generally occurs via a process called phagocytosis. Phagocytosis depends on the recognition of pathogen-associated molecular patterns (PAMP), e.g. flagellin and lipopolysaccharides (LPS) by specific receptors on the surface of macrophages (Buckner & Finlay, 2011; Mathur et al, 2012). Recognition of PAMPs allows the uptake of pathogens within a vesicular compartment, called the phagosome, into the inside of the cell. In the case of a *Salmonella* infection, the spacious phagosome shrinks to a tighter fitting vacuole, the SCV, over the time course of minutes to hours (Haraga et al, 2008). The SCV fuses with the lysosome, which contains antimicrobial peptides, reactive oxygen and nitrogen species, the latter generating the toxic gas nitric oxide (Steele-Mortimer, 2008). As outlined above, SPI-2 effectors have been shown to influence the fusion between the SCV and the lysosome, limiting the exposure to potentially bactericidal compounds.

At this stage, the further course of infection differentiates between typhoid and non-typhoid strains. Non-typhoid strains cause a localised inflammatory

response involving the local inflammation of the intestinal epithelium as well as the infiltration of PMN into the gut lumen (Haraga et al, 2008). Chemokines released by PMN increase the stress on epithelial cells and cause the loss of fluid and cells from the intestines through diarrhoea.

Typhoidal infections include the invasion of, as well as the multiplication within, macrophages followed by the spread of the bacteria via the reticulo-endothelial system, infecting organs such as the spleen, liver and gallbladder (Mastroeni, 2002).

In recent years, an increasing number of studies have reported the occurrence of invasive NTS (iNTS) strains across the world. It has been observed that not all strains are equally invasive in each country, hence strains particularly dominant in the United States are not necessarily the most important ones in European countries (Langridge et al, 2009a). Although they currently only contribute to a low number of NTS cases in America, they are a particular concern as they cause more severe infection than other NTS strains and have higher fatality rates (Jones et al, 2008).

In sub-Saharan Africa, cases of NTS presenting invasive symptoms have been increasing (Kingsley et al, 2009). Symptoms include bacteraemia, meningitis and septic arthritis rather than diarrhoea. A study by Kingsley *et al*. has shown that the genome of iNTS strains is degraded and that they are more similar to the genome of *S*. Typhi than before. Several risk factors such as malnutrition, anaemia, HIV infection and malaria make children particularly vulnerable, resulting in fatality rates between 20% and 25% (Gordon et al, 2008). In adults, fatal iNTS cases are closely associated with HIV infection and result in nearly 50% fatality (Gordon et al, 2002). In combination with the emergence of multi-drug resistant strains, this form of Salmonellosis poses an increasing threat (Kariuki et al, 2010).

1.2.3 Typhoid fever

Typhoid fever is characterised by the systemic spread of *S*. Typhi or *S*. Paratyphi serovars from the lymph nodes into the bloodstream, dissemination to organs such as the liver, colonisation of the gall bladder, discharge of bacteria

into the intestines via bile, secretion of bacteria in faeces and secondary infection of the intestine that leads to potentially fatal tissue damage (Figure 4).

Once the bacteria have reached the small intestine, they are taken up by M cells whose physiological function is to filter the gut lumen for foreign antigen or they induce uptake (Figure 5). M cells allow the passage of foreign particles through the epithelium into the Peyer's patches, where they are taken up by macrophages. Peyer's patches are gut-associated lymphoid tissue, functioning to recognize and present antigens as part of the intestine's immune system, initiating the adaptive immune response (Gill et al, 2011).

Removal from the gut lumen allows *Salmonella* to avoid the antimicrobial activity of the bile salts, low pH values, secreted immunoglobin A and digestive enzymes. Consequently, *Salmonella* are transported to the mesenteric lymph nodes, where they are able to multiply within phagocytic cells (Everest et al, 2001; Parry et al, 2002). The *Salmonella*-induced apoptosis of phagocytic cells leads to the release of bacteria into the bloodstream. Within two hours, the majority of *Salmonella* cells are cleared from the bloodstream. The complement factor C1q binds to the rough- and smooth-types of LPS on the surface of the bacteria, allowing for the recognition and internalisation of *Salmonella* into macrophages, dendritic cells and PMN (Mastroeni, 2002). Within the immune cells, *Salmonella* replicates whilst the immune cells transport it to the liver and spleen.

They re-enter the bloodstream, causing a secondary infection. Most clinical symptoms become apparent after this second round of infection approximately two weeks after the initial infection, when *Salmonella* infects the gall bladder, liver, spleen, bone marrow and re-infect the intestine (Everest et al, 2001; Parry et al, 2002). The infiltration of bone-marrow derived macrophages (BMDM) in the spleen and liver results in BMDM-rich granulomas causing organ swelling (Mastroeni, 2002). Re-infection of the intestine causes inflammation, ulceration and necrosis of the tissue. Inflammation is enhanced by the increased release of cytokines as a result of the re-exposure of the immune system to the pathogen, which may lead to necrosis (Parry et al, 2002).

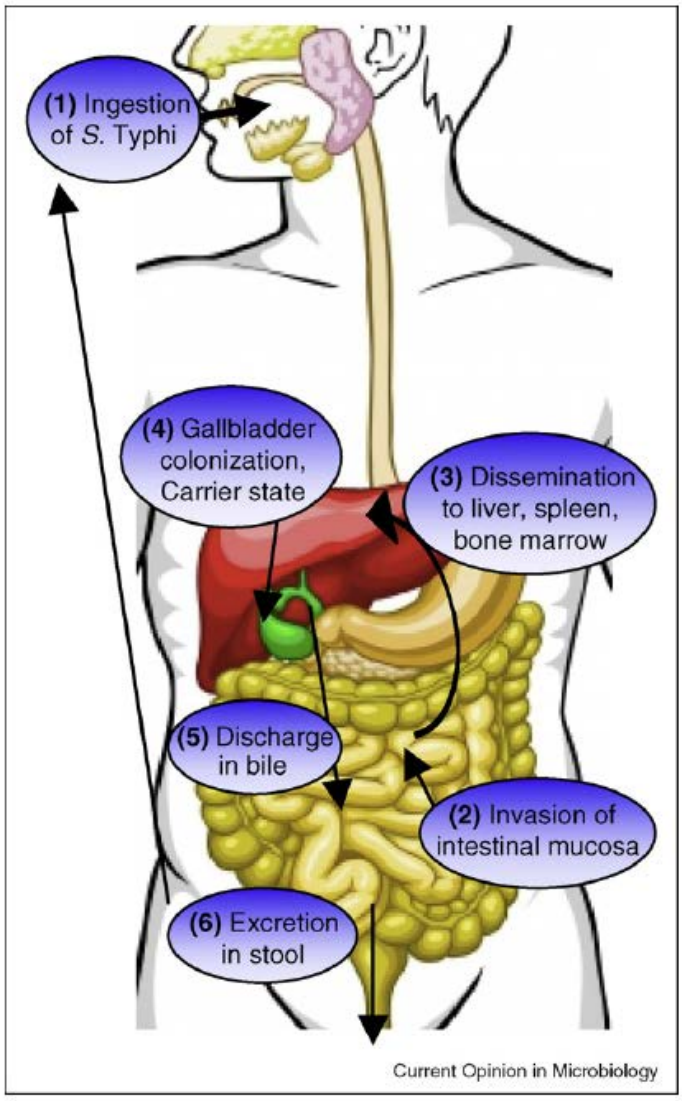

Figure 4 : Infection cycle of *S*. Typhi and chronic infective state (Tischler & McKinney, 2010)

Salmonella is taken up by ingestion (1) and reaches the small intestine after passing through the stomach. The invasion of the mucosa of the small intestine (2) and passage through M cells allow the uptake of bacterial cells into phagocytic immune cells. Due to several detoxification mechanisms, *Salmonella* survives the antibacterial activity of macrophages and multiplies within these immune cells. This is followed by the spread throughout the reticulo-endothelial system via the bloodstream (3), where *Salmonella* reaches organs such as the liver and the spleen as well as the bone marrow. Once it has colonised the gall bladder possibly causing the establishment of the chronic carrier state (4), bacteria re-enter the intestine via the discharge of bile through the bile duct (5). This shedding of bacteria into the intestine causes a secondary infection, leading to intestinal inflammation and damage to the blood vessels supplying the Peyer's patches. Perforation and haemorrhage from damaged tissue lead to peritonitis and septicaemia. Secretion of *Salmonella* via faeces (6) is the possible cause of infection of another or the re-infection of the same individual via the faecal-oral route.

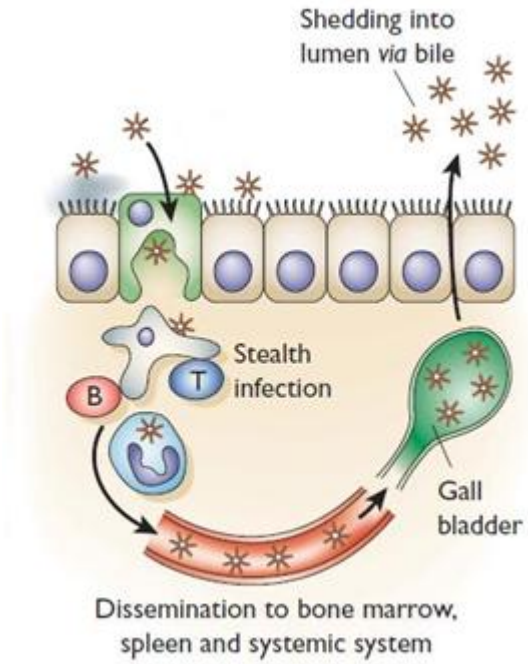

Figure 5: Intestinal entry of *S*. Typhi and route of re-infection (Young et al, 2002)
S. Typhi reaches the basolateral surface of the intestinal epithelial layer through passage or invasion of M cells. They are phagocytised by, for example, macrophages and are disseminated to the bone marrow, spleen and liver via the blood stream. When they reach the gall bladder, bacteria can re-enter the intestine via the discharge of bile through the bile duct. This shedding of bacteria causes a secondary infection, leading to intestinal inflammation and damage to the blood vessels supplying the Peyer's patches. Perforation and haemorrhage from damaged tissue can lead to peritonitis and septicaemia.

These more severe symptoms are only observed around three weeks after the initial infection with *Salmonella*. In severe cases, blood vessels that supply the Peyer's patches perforate, causing internal bleeding or perforation of ulcerated tissue occurs. Inflammation of the peritoneum or septicaemia by *Salmonella* is then potentially life-threatening. Without treatment within 96 hours of the first symptoms, between 60% and 90% of the infected patients die, whereas treatment with antibiotics such as Ciprofloxacin reduces the fatality rate to values below 5% (Everest et al, 2001; Parry et al, 2002).

S. Typhimurium infection of mice is used as a murine typhoid model as this serovar causes a systemic infection in mice, similar to *S*. Typhi observed in humans. Additionally, mice with different genotypic backgrounds can be used, allowing investigation into the cause of infection if, for example, the host immune system cannot produce NO or if it lacks the enzyme phagocytic oxidase Phox, also called NADPH oxidase that generates oxygen radicals. These animals can also be used to develop respective macrophage cell lines. Such models have highlighted that *S*. Typhimurium strains without SPI-2 genes are not able to replicate and survive within macrophages (Cirillo et al, 1998; Gallois et al, 2001).

In addition, the colonization of liver and spleen has been shown to be impaired for deletion strains (Silva et al, 2012). Therefore, SPI-2 is required to establish persistent infection. Comparative studies using *S*. Typhi in human macrophages and *S*. Typhimurium in mice, however, have highlighted phenotypic differences (Forest et al, 2010).

The Toll-like receptor TLR11 enables the recognition of *S*. Typhimurium in mice, but not *S*. Typhi, resulting in the inability of *S*. Typhi to infect mice (Mathur et al, 2012). The creation of a mouse TLR11 mutant has not only increased the susceptibility of the mice to *S*. Typhimurium infection, but also allowed *S*. Typhi to establish an infection.

1.2.4 Chronic carrier stage

After multiplication within the macrophage, typhoidal *Salmonella* spreads through the reticulo-endothelial system. This allows *Salmonella* to infect the spleen and the gall bladder of their host where they reside inside epithelial cells of the gall bladder or form biofilms on the surface of gall stones. Invading the epithelial cells as well as forming a biofilm allows *Salmonella* to remain undetected by the immune system (Tischler & McKinney, 2010). This stage can, in some patients, lead to either a chronic infection or to the perforation of tissue leading to a systemic wide spread of the bacteria resulting in peritonitis, sepsis and eventual death of the affected individual (Everest et al, 2001).

Chronic infections by *Salmonella* are characterised by the colonization of organs such as the gall bladder, gall stones, the liver or the spleen and affect humans, but systemic infection of pigs, chicken and other poultry is also common (Hohmann, 2001; Lai et al, 1992; Milnes et al, 2008). The serovar particularly associated with chronic infection in pigs is *S*. Choleraesuis (Rabsch et al, 2002). Carriers do not have any overt clinical symptoms, but often shed bacteria through their faeces as bacteria are secreted into the intestines with the discharge of bile. The lack of clinical symptoms poses a problem as human chronic carriers are often not aware of the potential contamination hazard they cause. Similarly, infected animals are not detected and their meat is then potentially used further for the production of food or their excrements are used to fertilise the soil for agricultural purposes. Such use of contaminated excrements causes the contamination of vegetables with *Salmonella* and provides another source of infection for human beings.

Although the chronic carrier itself is not at risk directly, a chronic infection should be closely monitored and avoided in the agricultural environment. Carriers should be treated to prevent the spread of *Salmonella* from animals to humans or from infected humans to other individuals. Shedding of bacteria through the faeces occurs for a prolonged period of time and antibiotic therapy is only effective if the infection is not localised within human tissue cells (Crawford et al, 2010). If the source of the chronic carriage has been located to gallstones, cholecystectomy is recommended.

1.3 Treatment of *Salmonella* infections

In case of an infection with non-typhoidal *Salmonella*, gastroenteritis is often a self-clearing infection and mostly does not require the administration of antibiotics in non-risk groups. In immunocompromised hosts; the elderly, children and patients with immune diseases, the use of antibiotics has to be considered. However, it has been shown that children infected with multi-drug resistant (MDR) NTS are more likely to need treatment for diarrhoea and bacteraemia (Kariuki et al, 2006; Varma et al, 2005).

Typhoid fever requires medical attention and is treated with antibiotics. The choice of treatment has changed over the years due to the emergence of multi-drug resistance and this limits the treatment options drastically. Chloramphenicol has first been used for treatment of typhoid fever and has been the antibiotic of choice until the 1980s (Anderson & Smith, 1972) when significant levels of resistance have been observed. Then, a combination of Ampicillin with Trimethoprim-Sulfamethoxazole has been introduced, although this also has been retracted due to increasing cases of resistance (Butler et al, 1977; McHugh et al, 1975). Since the 1990s, first- and second-generation quinolones (Nalidixic acid; Ciprofloxacin and Ofloxacin, respectively) have been used to treat typhoid fever patients (Uwaydah et al, 1991; Wang et al, 1989).

Another reason for concern regarding the emerging of drug-resistant strains has been reported: *S*. Typhi strains that have showed a decreased Ciprofloxacin susceptibility increase the patient's recovery time from the fever and also increase the number of cases where treatment failed (Crump et al, 2008).

1.4 Antibiotic resistance

Antibiotic resistance of *S.* Typhi strains has been reported in individuals since the 1950s and has resulted in a first outbreak caused by an endemic strain in Mexico in 1972 (Anderson & Smith, 1972). The particular strain has been shown to contain Vi phages types A or 46, known resistance factors originating from *E. coli* that confer Chloramphenicol resistance to the *S.* Typhi strain. Other epidemics caused by Chloramphenicol-resistant *S*. Typhi strains have followed in South-East Asia, highlighting the fast emergence of drug resistance and the danger for causing epidemics (Anderson, 1975). The use of Ampicillin and

Trimethoprim-Sulfamethoxazole has since been recommended, but Ampicillin resistance in *S*. Typhimurium has already been reported in the mid-1970s (McHugh et al, 1975). This has been followed by the identification of widely distributed multi-drug resistant *S*. Typhi and *S*. Typhimurium strains characterised by their resistance to Ampicillin, Chloramphenicol, Streptomycin, Sulfonamide and Tetracycline (ACSSuT) (Helms et al, 2005; Jesudasan et al, 1990; Ng et al, 1999; Threlfall et al, 1993; Threlfall et al, 1992). Three different phage-types of *S*. Typhimurium have been identified which resulted in ACSSuT resistance, including DT104. DT104 is the Definite type 104 that contains a P22-like phage insert and a 43 kb insertion of a *Salmonella* genomic island 1 (SGI1), containing genes for Ampicillin (*pse*), Chloramphenicol (*floR*), Streptomycin (*str* or *aad*), Sulfonamide (*sulI*) and Tetracycline (*tetG* or *tetR*) resistance (Chiu et al, 2006).

As a result, quinolones such as Nalidixic acid have initially been recommended for treatment, but cases of resistance have been reported following the isolation of strains from poultry and other animals (Griggs et al, 1994). Although second-generation fluoroquinolones like Ciprofloxacin have been in use, cases reporting resistant strains of *S*. Typhimurium isolated from humans have followed shortly (Piddock et al, 1993). Such incidences are reported regularly and usually involve mutations in genes encoding for the DNA gyrase (*gyrAB*) or the topoisomerase IV (*parCE*) (Chiu et al, 2002; Fey et al, 2000; Glynn et al, 2004). A new type of quinolone-resistant genotype mediated by *qnr* genes on a plasmid has also been reported (Gunell et al, 2009; Lindgren et al, 2009).

1.4.1 Vaccination

Currently, two types of licensed vaccines are available to protect against *S*. Typhi infection. One is a live attenuated vaccine based on *S*. Typhi Ty21a, the other is based on the administration of a conjugate vaccine containing the Vi antigen (Berna-Biotech, 2006; GlaxoSmithKline, 2009; Sanofi-Pasteur, 2009).

The live attenuated vaccine has been recommended for oral administration in acid-resistant capsules and four doses have to be taken on alternate days (Berna-Biotech, 2006). The vaccine efficacy has been determined to be 69% and protection of individuals aged six or higher against infection by *S*. Typhi

lasts for at least five years. The other type of vaccine is a conjugate vaccine containing the virulence capsular polysaccharide of *S*. Typhi (Vi) (GlaxoSmithKline, 2009; Sanofi-Pasteur, 2009). The vaccine is used to protect individuals from the age of two after a single injection, providing an efficacy rate of over 70%, but a booster is required every three years.

Comparing the indicators for successful immunization of individuals, several points have to be noticed: Firstly, both vaccines do not offer protection for children under the age of two years. However, babies in particular are at risk of death from typhoid fever. Secondly, in rural areas of sub-Saharan Africa, where the incidence rate of typhoid fever is high, access to health care is often limited and and booster vaccinations are not practical. Thirdly, refrigeration of vaccines is particularly difficult in the non-moderate climate of sub-Saharan Africa. Lastly, the vaccines only confer protection against the infection by *S*. Typhi, but do not protect against *S*. Paratyphi A, B or C infections. Therefore, there is potential for the improvement of currently available vaccines to cover more serovars causing enteric fever, to allow storage at higher temperatures as well as to reduce the need of booster vaccinations due to impracticality.

Research for the discovery of new vaccines and antibiotics is needed as treatment of typhoid fever and iNTS become more difficult due to a rise in multi-drug resistance in *Salmonella*. One possibility is the use of oral live attenuated strains that are administered via the natural route, elicit a protective immune response, but have minimal side effects. Potential new strains have been created over the years that have the potential to either be used as live oral vaccines or to serve for large-scale purification of potential antigens. One example is the *S*. Typhi CVD908-*htrA* strain developed by Chatfield and colleagues (Chatfield et al, 1993; Tacket et al, 2000; Tacket et al, 1997). It lacks the gene for the heat-shock protein HtrA as well as the genes *aroC* and *aroD*, involved in amino acid synthesis. Clinical trials of phase I and II have shown that the strain has very low toxicity, but sufficiently expresses e.g. the fragment C of tetanus toxin to elicit effective and protective levels of antibodies against the tetanus toxin after a single oral dose (Tacket et al, 2000). This strain is then also used to express *Salmonella*-specific proteins for the use as a Typhoid fever or NTS vaccine. More recently, a *S*. Typhi Ty2 *aroC*$^{-}$ *ssaV*$^{-}$ strain has been used

in a study with human volunteers (Kirkpatrick et al, 2006). With the deletion of the SPI-2 gene *ssaV* and the *aroC* important for aromatic biosynthesis, systemic spread of *S*. Typhi is prevented and oral administration of a single dose is well tolerated by the volunteers. In addition, significant levels of *S*. Typhi LPS IgA have been measured, making this strain a promising candidate for future vaccines.

Although NTS do not cause typhoid fever, in combination with other infections such as Malaria or HIV, NTS-infected patients have an increased mortality rate from bacteraemia (Andrews-Polymenis et al, 2010). This has resulted in the search for possible vaccine strains, not only against *S*. Typhi and *S*. Paratyphi, but also including *S*. Typhimurium and *S*. Enteritidis.

Tennant *et al*. chose two NTS serovars that account for the highest number of infections in Africa as well as for a high proportion of gastroenteritis in Europe and Northern America (Tennant et al, 2011). They deleted up to four genes from the genomes of *S*. Typhimurium and *S*. Enteritidis strains. The choice of genes is based on previous reports of attenuated virulence of deletion strains in mice or human infection models. *GuaBA* are two genes that are important for guanine synthesis and it has been shown previously that their deletion results in decreased virulence of *Shigella flexneri* strains as well as *S*. Typhi (Kotloff et al, 2004; Wang et al, 2001). Similar, deletion of either protease *clpP* or *clpX* causes attenuation of *S*. Typhimurium and provide protection to mice after oral administration (Matsui et al, 2003). Additionally, Δ*clpP* or Δ*clpX* strains are characterised by hyperflagellation, which increases the display of antigens on the surface of the bacterial cell (Tennant et al, 2011). A combination with the deletion of *fliD* results in the overproduction of flagellin monomers. This supports the use of such a strain for the large-scale purification of antigens that are necessary components of live vaccines. *S*. Typhimurium strains with deletions of *clpP* and *guaBA* or a combination with the deletion of *fliD* are highly attenuated during mice infection. The double deletion strain of *S*. Enteritidis Δ*guaBA clpP* also shows decreased virulence. These three strains are able to decrease bacterial shedding in mouse faeces and provide protection against otherwise lethal doses of *S*. Typhimurium and *S*. Enteritidis, respectively. It has been shown that the mice develop high levels of immunoglobin G (IgG) against

lipopolysaccharides and flagella antibodies. The vaccine efficacy is around 80% for all three strains and the next steps for further clinical trials have been initiated.

Previously a range of deletion strains has been shown to be attenuated during mouse infection and has been proposed for the use as live vaccines, including Δ*aroA* and Δ*phoP* (Galán & Curtiss, 1989b; Hormaeche et al, 1990; Hormaeche et al, 1991). However, due to low immunogenicity, and reports regarding side effects, their use has not advanced to the final approval for human use (Angelakopoulos & Hohmann, 2000; Hindle et al, 2002). Therefore, there are currently no live oral vaccines on the basis of NTS on the market.

Another study has also employed the use of *S*. Enteritidis Δ*guaBA clpP*, but has concentrated their experiments on the constructions of flagellin and polysaccharides to form the basis of a conjugate vaccine (Simon et al, 2011). Similar to the study using strains as live vaccines, they have achieved an increased immune response when compared to using flagellin on its own and have proven a vaccine efficacy between 80% and 100%.

Several studies have used O polysaccharides (OPS) for enhanced immunogenicity of conjugate vaccines against *S*. Typhimurium strains in the past (Svenson et al, 1979; Watson et al, 1992). Similar, OPS of *S*. Paratyphi has shown promising response in Phase I and II clinical trials (Konadu et al, 1996; Konadu et al, 2000). Improving the Vi-containing vaccine to prevent typhoid fever has been the task of several groups and promising results using several combinations of Vi with different protein carriers have been demonstrated. Tetanus or Diphtheria toxin as well as the *Pseudomonas aeruginosa* recombinant exoprotein A (rEPA) and a non-toxic variant of diphtheria toxin (CRM_{197}) have been used in the past (Cui et al, 2010; Kossaczka et al, 1999; Lin et al, 2001; Mai et al, 2003; Micoli et al, 2011). These toxins and proteins are used to create conjugate vaccines to increase the immunogenicity of the low immunogenic Vi-component that functions to prevent complement recognition of *S.* Typhi (Wilson et al, 2011).

Nevertheless, most of these strains are a long way from being used for immunization of humans and do not address the problem of reducing or eliminating the needs for booster injections.

The need for vaccination of humans is followed by the need to prevent chicken populations from becoming reservoirs for the spread of *Salmonella*. Hence, studies supporting the development of vaccines for hens based on live or killed attenuated strains of *S*. Typhimurium and *S*. Enteritidis have been reported (Babu et al, 2003; Curtiss III & Hassan, 1996; Holt et al, 2003). In the UK, The Lion Code of Practice has been introduced in 1998 and includes the mandatory vaccination of laying hens against *S*. Enteritidis for farmers that are members of the British Egg Industry Council. In combination with more stringent controls of hygiene and more frequent testing for *Salmonella*, the Lion Quality mark is printed onto the egg shell and box to indicate eggs produced under this scheme. The number of eggs produced in the UK coming from a business that is part of this scheme has risen to 95% in 2010. The number of cases of food-poisoning arising from *S*. Enteritidis has fallen and this has been attributed to the vaccination programme in combination with stricter hygiene and health measures on farms and during food production (O'Brien, 2013).

1.5 Immune defence: Macrophages

A range of different immune cells and processes belong to the innate immune system, which is the first line of response to the entry of foreign antigens into the human body. Those cells include, for example, dendritic, mast and phagocytic cells such as macrophages (Tripathi et al, 2007). Their roles vary from the detection of antigens, the activation of lymphocytes to phagocytosis and killing of bacterial pathogens as a general response to the entry of pathogenic bacteria.

As described previously, macrophages are of major importance for the progress of *Salmonella* infection. *Salmonella* invades intestinal epithelial cells to avoid recognition by the immune system. Another way to avoid detection in the lumen of the intestine is to reach the basolateral surface by passing through M cells. This is followed by recognition of *Salmonella* by macrophages, which then induces the phagocytosis of the bacterial cells (Haraga et al, 2008). The bacteria are first contained in a vesicle called a phagosome that matures into the SCV before the fusion with a lysosome occurs. Several antibacterial mechanisms then become activated. Firstly, ROS are generated by the NADPH

phagocyte oxidase encoded by the *phox* gene. Secondly, nitric oxide is generated by the inducible nitric oxide synthase iNOS (Fang, 2004). iNOS is specific to macrophages and is not constitutively expressed (Tripathi et al, 2007). Two other isoforms of nitric oxide synthase (NOS) exist. Both isoforms are calcium- and calmodulin-dependent and are found in neurons as well as in epithelial cells and hepatocytes respectively (Burgner et al, 1999). All three isozymes oxidize L–arginine producing nitric oxide and L–citrulline by using electrons from the electron donor NADPH (Wang & Ruby, 2011). Homologues to the eukaryotic NOS have been found in Gram-positive bacteria as well as Archaea, where they have been implicated in stress and growth responses (Crane et al, 2010).

Free radicals such as NO and hydrogen peroxide damage the bacterial DNA as they deaminate the nucleotides and the radicals further react with oxygen or one another to form other toxic compounds such as the bactericidal peroxynitrite (Linehan & Holden, 2003; Wink et al, 1991). In addition, lysosomal enzymes and defensins are present within the phago-lysosome, aiding in the bactericidal activity of the reactive nitrogen and oxygen species (Mastroeni, 2002). The acidification of the phago-lysosome poses an additional risk for bacteria; however, *Salmonella* has been shown to delay this process using changes in gene expression via the PhoPQ two-component system (Alpuche Aranda et al, 1992).

Nitric oxide is a free radical that has been found to have several functions within the human body and in the environment. It is lipophilic and small in size and hence accumulates in membranes and is able to enter through as well as cross them (Hughes & Robert, 2008). These properties allow it to function as a neuronal signalling molecule as well as to help regulate blood pressure through the vasodilation of smooth muscle cells found on blood vessels (Poole & Hughes, 2000; Schoedon et al, 1995). The other two isoforms of NOS, namely neuronal NOS (nNOS) and endothelial NOS (eNOS), support this function by ensuring the site-directed production of NO (Burgner et al, 1999; Hughes & Robert, 2008). Although it is a radical, it is rather stable, but it still reacts with other radicals. In the environment, this reactivity increases the destruction of ozone, as both NO and nitrous oxide damage the stratosphere (Martinez-

Espinosa et al, 2011). However, the reactivity of NO has also been shown to provide protection against oxidative and UV stress (Wang & Ruby, 2011). Its characteristics as a signalling molecule are not limited to mammals, but have also been shown for plants as well as squids (Dordas et al, 2003; Ferrarini et al, 2008; Wang et al, 2010a; Wang et al, 2010b).

The focus here is on NO as a component of the mammalian immune system and its antimicrobial activity. Within macrophages, iNOS is found within the cytosol as well as within intracellular small vesicles (Fang, 2004). Activity of iNOS is tightly regulated by a number of cytokines such as interferon gamma (IFN-γ), tumour necrosis factor alpha (TNF-α), IL-2 and IL-6 (De Groote & Fang, 1995).

1.5.1 Toxicity of reactive oxygen species

In order to be able to survive the antibacterial activity within macrophages, *Salmonella* ssp. have evolved detoxification mechanisms to counteract the bactericidal environment. To prevent cell death, *Salmonella* can employ several protection mechanisms to avoid being targeted by reactive oxygen and nitrogen species. Mechanisms for the detoxification of reactive oxygen species that are released during the oxidative burst occurring after one to two hours post uptake into the SCV have been intensively studied and major mechanisms have been identified, which are discussed below (Tsolis et al, 1995; Vazquez-Torres et al, 2000a).

The reduction of oxygen by the NADPH oxidase leads to the production of superoxide anions (O_2^-), which form hydrogen peroxide (H_2O_2) upon dismutation (Vazquez-Torres & Fang, 2001a). Both hydrogen peroxide and superoxide anions cause damage to iron-sulphur ([Fe-S]) clusters (Imlay & Imlay, 2002). In addition, H_2O_2 also oxidizes thiol groups and in combination with Fe(II) leads to the formation of hydroxyl radicals (OH^-), hydroxide ($OH^\cdot$) and Fe(III) in the so-called Fenton reaction. Hydroxyl radicals react with a range of molecules, including DNA, and DNA damage is believed to be closely linked to this chemical reaction (Woodmansee & Imlay, 2003).

[Fe-S] clusters are present in a wide range of proteins and damage to these structures causes a change in the confirmation of the proteins, making them

prone to protein degradation due to their misfolded structure and inhibiting enzymatic reactions since the active centre is no longer functional (Imlay & Imlay, 2006). Another effect notable for transcription factors is the fact that a change of confirmation can cause a change in DNA binding properties and consequently altering gene expression levels positively or negatively, either through alleviated repression or through targeting a different DNA recognition motif. Reactivity of the protein-bound iron with e.g. NO has been shown to cause such regulatory changes for FNR- and NorR-regulated genes (Baptista et al, 2012; Cruz-Ramos et al, 2002).

1.5.1.1 Enzymatic detoxification of ROS by *Salmonella*

There are several different protection mechanisms against free radicals that have been discovered in *Salmonella.* One of them is the production of enzymes, e.g. superoxide dismutases or hydroperoxidases that convert the toxic radicals into less toxic compounds (Gilberthorpe et al, 2007). Two Cu,Zn superoxide dismutases (Cu,ZnSOD) are present in *S.* Typhimurium and one of them (SodC1) is closely linked to pathogenesis (Pacello et al, 2008). In combination with the ferroxidase activity of a DNA-binding protein called Dps, these three enzymes prevent the cytotoxic activity of hydrogen peroxide in the peri- and cytoplasm (SodC1/SodC2 and Dps, respectively). In addition, a catalase-peroxidase (KatG) first found in *Mycobacterium tuberculosis* also exerts peroxynitritase activity in *S.* Typhimurium, highlighting its potential role in the detoxification of oxidative stresses (McLean et al, 2010). Another mechanism of protection involves the expression of the TTSS SPI-2, which prevents the trafficking of Phox-containing vesicles to the phagosome. Various deletion mutations of genes in the SPI-2 region result in strains that are less virulent in macrophage and mice experiments (Gallois et al, 2001; Vazquez-Torres et al, 2000b). Wild-type strains are nevertheless exposed to oxidative stress as they are not able to prevent the fusion of Phox-containing vesicles with the SCV completely, but both research groups show that the percentage of localisation has been lowered by ~ 40%.

The importance of Phox in preventing infection by pathogens has been highlighted in several mice infection experiments: An infection of mice lacking the *phox* gene (C57BL/6 gp91*phox*$^{-/-}$), which are therefore unable to produce an

oxidative burst, leads to the death of the animals after a few days, whereas wild-type mice (C57BL/6) are able to clear the infection caused by *S.* Typhimurium (Mastroeni et al, 2000). Bone marrow derived macrophages extracted from *phox*-deficient mice are not able to suppress the bacterial growth, resulting in a survival rate of the wild-type *Salmonella* of nearly 100% (Vazquez-Torres et al, 2000a). In a similar manner, another research group has observed the diminished capacity of *phox*-deficient mice to withstand the infection by a virulent wild-type and the attenuated mutant strain Δ*recBC* (Shiloh et al, 1999). *In vitro* experiments using macrophages derived from wild-type and *phox*-deficient mice show that the lack the oxidative burst reduces the capacity of the macrophages to kill *E. coli*, *Listeria monocytogenes* and *Salmonella* wild-type and attenuated strains significantly.

1.5.2 Toxicity of reactive nitrogen species

Reactive nitrogen species (RNS) provide a second line of antimicrobial defence for the immune system. RNS include a number of different chemical compounds, such as nitric oxide (NO), peroxynitrite ($ONOO^-$), nitrosothiols (SNO) and nitrous acid (HNO_2). RNS interferes with a number of biological molecules and structures, including, but not limited to: i) protein-bound metals; ii) amino acid biosynthesis and transport; iii) DNA strands and iv) transcriptional regulators (Fang, 2004).

For *Salmonella* pathogenicity, RNS become important at two different stages during infection, firstly in the mouth and stomach and secondly within the phagosome of macrophages. The salivary glands harbour nitrate that is reduced to nitrite by oral commensal bacteria (Bourret et al, 2008). A direct correlation between the dietary intake of nitrite and nitrate concentrations in salivary glands has been shown (Gilchrist et al, 2010; Spiegelhalder et al, 1976). The nitrate in the saliva then serves as an electron acceptor for anaerobic respiring bacteria in the mouth and the by-product nitrite reaches the stomach upon swallowing. In combination with the acidity of the gastric juice, nitrite then protonates to form acidified nitrite, also referred to as nitrous acid (HNO_2) (Benjamin et al, 1994). Nitrous acid dissociates to form not only nitric oxide, but also dinitrogen trioxide (N_2O_3) and nitrogen dioxide (NO_2). Inhibition of growth and decreased oral virulence by acidified nitrite and other RNS have

been demonstrated for *S*. Typhimurium (Bourret et al, 2008; Kim et al, 2003). Similar studies have shown the susceptibility of *E. coli* and *Candida albicans* to nitrite after acid exposure in a gastric juice containing stomach model (Benjamin et al, 1994; Björne et al, 2006). Again, a strong correlation between nitrite, nitrate and NO concentrations in the stomach and dietary intake of nitrate is observed (Björne et al, 2006; McKnight et al, 1997). Concentrations of these nitrogen compounds can increase up to ten times after a nitrate solution has been consumed. Such changes in concentrations pose a significant risk to pathogens and stress the important role of dietary nitrate in the defence against pathogens. Although *Salmonella* mounts the acid-tolerance response to protect itself against the low gastric pH, gastric RNS have been shown to inhibit the response by interfering with the two-component system PhoPQ in an unidentified mechanism (Bourret et al, 2008). Together, high nitrate concentrations in the oral cavity, high acidity of the gastric juice and RNS provide a highly bactericidal barrier against infection. However, the high number of cases of infections by e.g. *Salmonella* and enteropathogenic *E. coli* indicate that this is a sufficient barrier against some, but certainly not all intestinal pathogens.

To further reduce the number of pathogenic bacteria, macrophages mount a second line of defence employing RNS. The release of nitric oxide during the nitrosative burst into the phagosome has been determined to occur approximately six to eight hours after phagocytosis in mice and might occur earlier within human macrophages (Eriksson et al, 2003; Stevanin et al, 2002; Vazquez-Torres et al, 2000a). In addition, release of N_2O_3 has been demonstrated (McCollister et al, 2008). RNS readily react with metals, such as iron and zinc, which are often protein-bound (Schapiro et al, 2003). As a result, Fe(II) is released from [Fe-S] clusters and accelerates the Fenton reaction that causes the release of ROS (Fang, 2004). It also inhibits bacterial respiration by binding reversibly to two different cytochrome oxidases in *E. coli*, further accentuating the antimicrobial activity of RNS in the phagosome (Butler et al, 1997; Hori et al, 1996; Woodmansee & Imlay, 2003). In contrast, it has been proposed that the inhibition of respiration leads to the accumulation of NADH, which in turn scavenges $OH^{\cdot}$ and results in increased resistance to H_2O_2 in *Salmonella* spp. (Husain et al, 2008).

Synergy between ROS and RNS also results in the formation of peroxynitrite that like NO has been implicated in causing DNA damage ((Szabó et al, 1996), reviewed in (Fang, 2004) and (Bogdan et al, 2000)). Interference with ribonucelotide reductase also means that DNA cannot be synthesized or repaired, accentuating the DNA damage already caused by NO (Fang, 2004). In addition, NO inhibits the regulation of DskA that controls the biosynthesis and transport of amino acids, disrupting the metabolic functioning of the cell (Henard & Vázquez-Torres, 2012). Peroxynitrite has also been shown to be a precursor for the generation of nitrate, *S*- and *N*-oxides in the gut lumen as the result of reactivity of ROS with RNS and oxidation of organic sulphides and tertiary amines (Winter et al, 2013). These three compounds are used as a terminal electron acceptor in anaerobic respiration by Enterobacteriaceae, providing *E. coli* and *Salmonella* with a competitive advantage over fermenting microbes of the intestinal flora. Furthermore, ROS react with thiosulphate to generate tetrathionate. Thiosulphate is created through the detoxification of H_2S that is produced by colonic bacteria. Tetrathionate also serves as an electron acceptor and growth advantage of *S*. Typhimurium has been demonstrated (Winter et al, 2010). Therefore, *Salmonella* exploits the secretion of ROS and RNS in the gut lumen to provide itself with an additional source of electron acceptors needed for anaerobic respiration.

Interference of RNS with proteins by causing *S*-nitrosation is wide-spread and for example affects the citric acid cycle involved in energy metabolism (Richardson et al, 2011). Nitrosation also affects the regulation of NO detoxification genes. Transcriptional regulators such as FNR, Fur and NsrR contain [Fe-S] clusters. The iron reacts with NO and forms iron-nitrosyl complexes that no longer allow for DNA binding (Cruz-Ramos et al, 2002; D'Autréaux et al, 2002; Isabella et al, 2009). Therefore, this allows the expression of genes such as *hmpA* encoding for a flavohaemoglobin that converts NO to nitrate as a means to detoxify NO.

1.6 Detoxification of NO

Defence against RNS follows three different routes: RNS either is enzymatically detoxified, the radicals are scavenged or the damage is repaired. During infection, all three possibilities are employed by *Salmonella* to ensure survival. Particular attention is given to proteins that allow the enzymatic detoxification of NO.

Such enzymes are not unique to pathogenic bacteria such as *E. coli* and *Salmonella*, but are also found in denitrifiers. During anaerobic conditions, these bacteria and archaea use nitrate and nitrite as electron acceptors to maintain their metabolism (Arkenberg et al, 2011). Several enzymes are necessary to perform complete denitrification, forming part of the microbial nitrogen cycle as shown in Figure 6 (Watmough et al, 1999). Firstly, nitrate is reduced to nitrite by either an membrane-bound (Nar) or periplasmic form of a nitrate reductase (Nap) (Zumft, 1997). The gene transcription is linked to the change from aerobiosis to anaerobiosis as oxygen levels serve as a regulator for gene expression. The enzyme consists of complexes with two to three subunits and contains a [Fe-S] binding site and a molybdenum co-factor. A channelling of nitrite from a membrane-bound form of nitrate reductase into the periplasmic space has been proposed, but remains to be further elucidated. Three types of nitrite reductases have been identified, but only one type per species has been found (Watmough et al, 1999). *Paracoccus denitrificans* and *Pseudomonas stutzeri* possess a cytochrome cd_1 nitrite reductase encoded by the *nirS* gene. It forms a homodimer and has two different haem groups. The copper nitrite reductase of *Rhodobacter sphaeroides* 2.4.3 encoded on the *nirK* gene is an example for the second type of nitrite reductase, but forms a trimer with two different domains. This enzyme family has three sub-classes, but all sub-classes require copper to form a Cu^{+}-NO^{+} complex during nitrite reduction (Zumft, 1997). The third class of nitrite reductase is the cytochrome *c* nitrite reductase. Rather than reducing nitrite to NO, it produces ammonia. It is also located within the periplasm and has 5 *c*-type haems. Both the nitrite reductases and the nitric oxide reductase are located within the periplasm. This prevents the diffusion of NO into the cytosol as it is reduced to the less toxic nitrous oxide without any need of a channelling protein.

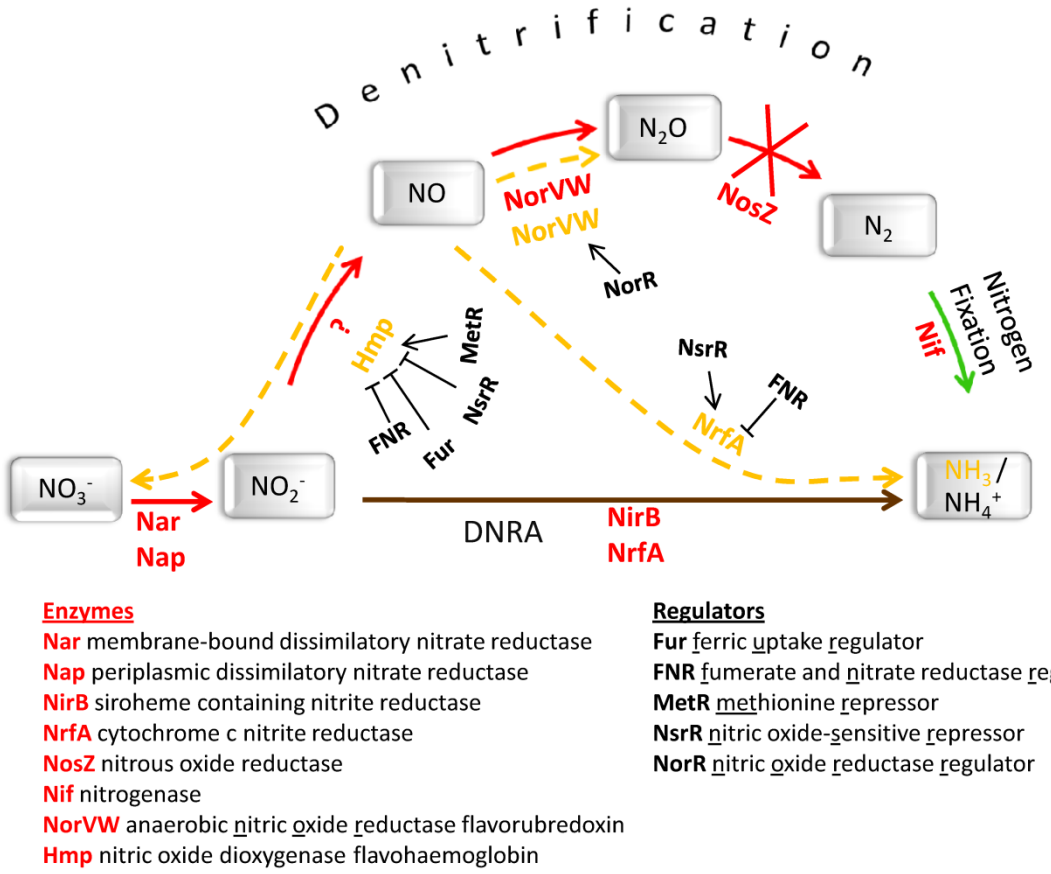

Figure 6: Truncated denitrification pathways in *E. coli* and *S.* Typhimurium (Arkenberg et al, 2011)

Nitrate respiration in *E. coli* and *Salmonella* is a truncated version of the denitrification pathway (red arrows). Unlike many soil bacteria, *E. coli* and *Salmonella* lack NosZ; indicated by a red cross. Nitric oxide (NO) is a toxic intermediate. The main enzymes involved in NO detoxification alongside their regulators are shown. The NO detoxification pathways are indicated by yellow dashed arrows and enzymes involved in these pathways are shown in yellow. Positive regulation is highlighted by arrows and negative regulation by perpendicular lines. Other enzymes are shown in red.

In addition, superoxide dismutase is present in the periplasm, which reduces the amount of hydroxyl radicals that potentially react with NO to form peroxynitrite. Nitric oxide reductases are membrane-bound due to their strong hydrophobicity and their active centre faces into the periplasm. The enzyme consists of the subunits NorB and NorC and has two haems. Haems are also present in the nitrous oxide reductase, encoded by the *nos* genes. It has two identical subunits and each contains a binuclear copper centre. The dinitrogen formed by the activity of the nitrous oxide then readily diffuses out of the cell.
Denitrifiers such as *P*. *denitrificans* and *P*. *stutzeri* are exposed to a steady-state concentration of NO, ranging from 20-50 nM. Therefore, it is important for the survival of the bacteria to be able to detoxify NO into N_2O, followed by the conversion into dinitrogen. Tight regulation of the nitrate and nitrite reductases by nitrate as well as NarL and FNR-like factors such as NnrR and NNR has been shown that limits the expression of the reductases to oxygen-limited and anaerobe environments (Zumft, 1997). The properties of the regulator FNR are further discussed in 1.7.1. The limited amount of oxygen in the periplasm reduces the potentiated toxicity of NO and in addition NO also directly regulates the activity of the nitric oxide reductase.

Although *Salmonella* is not a primarily soil-dwelling bacterium, it possesses the ability to perform a truncated version of denitrification. It uses nitrate and nitrite as electron acceptors during anaerobic growth also when residing in the gastrointestinal tract, leading to the production of NO and N_2O, but it is lacking the nitrous oxide reductase to further convert N_2O into dinitrogen (Winter et al, 2013). So far, no enzyme with nitrous oxide reductase activity has been discovered in bacteria of the Enterobacteriaceae family. However, nitrous oxide is less toxic than NO and diffuses out of the bacterial cell. The enzymes and regulators involved in the truncated denitrification of *Salmonella* and *E. coli* are shown in Figure 6. Both bacteria have three nitrate reductases, two membrane-bound systems encoded in the *nar* operons *narGHJI* and *narZYWV* as well as a periplasmic type encoded by *napFDAGHBC* (Zumft, 1997). Both types of enzymes contain [Fe-S] clusters and a molybdenum co-factor (Bertero et al, 2005; Jepson et al, 2007; Jormakka et al, 2004).

Although NarG and NarZ seem to be isozymes, their regulation differs as NarZ is active during aerobic growth, whereas NarG is predominantly expressed during anaerobic conditions (Prior et al, 2009; Rowley et al, 2012). Nap is active during anaerobic conditions, where nitrate concentrations are low and Nap is then the main contributor to ammonia production coupled to the activity of the cytochrome *c* reductase NrfA. NarG is regarded as the main contributor to nitrate reduction with up to 98% attributed to its activity when nitrate concentrations are high. Several anti-porters are found in the periplasmic membrane to allow either the transport of nitrate into the cytoplasm and nitrite into the periplasm (NarK, NarU), or the transport of nitrite into the cytoplasm and the transport of protons into the periplasm (NirC) (Rowley et al, 2012; Vine & Cole, 2011). A schematic of the different locations of the enzymes within the cell are shown in Figure 7.

Nitrite is reduced to ammonia by NrfA in the periplasm, by the nitrite reductase NirB in the cytoplasm or it is reduced to yield nitric oxide. Additionally, NarG has been shown to produce NO from nitrite once the nitrate pool has been exhausted (Gilberthorpe & Poole, 2008). The further reduction of NO to produce nitrous oxide is conducted by NorVW and further discussed in 1.6.2. So far, no enzyme with nitrous oxide reductase activity has been discovered in bacteria of the Enterobacteriaceae family, therefore truncating the denitrification cycle at the stage of nitrous oxide. Nitrous oxide emissions are measured in the head space of anaerobic cultures and previous studies have shown that all the nitrogen provided by nitrate in the medium has either been converted to nitrite or to nitrous oxide (Rowley et al, 2012). As nitrous oxide is the result of the reduction of toxic NO, the concentration of nitrous oxide serves as an indicator for the nitric oxide concentration in the medium and headspace.

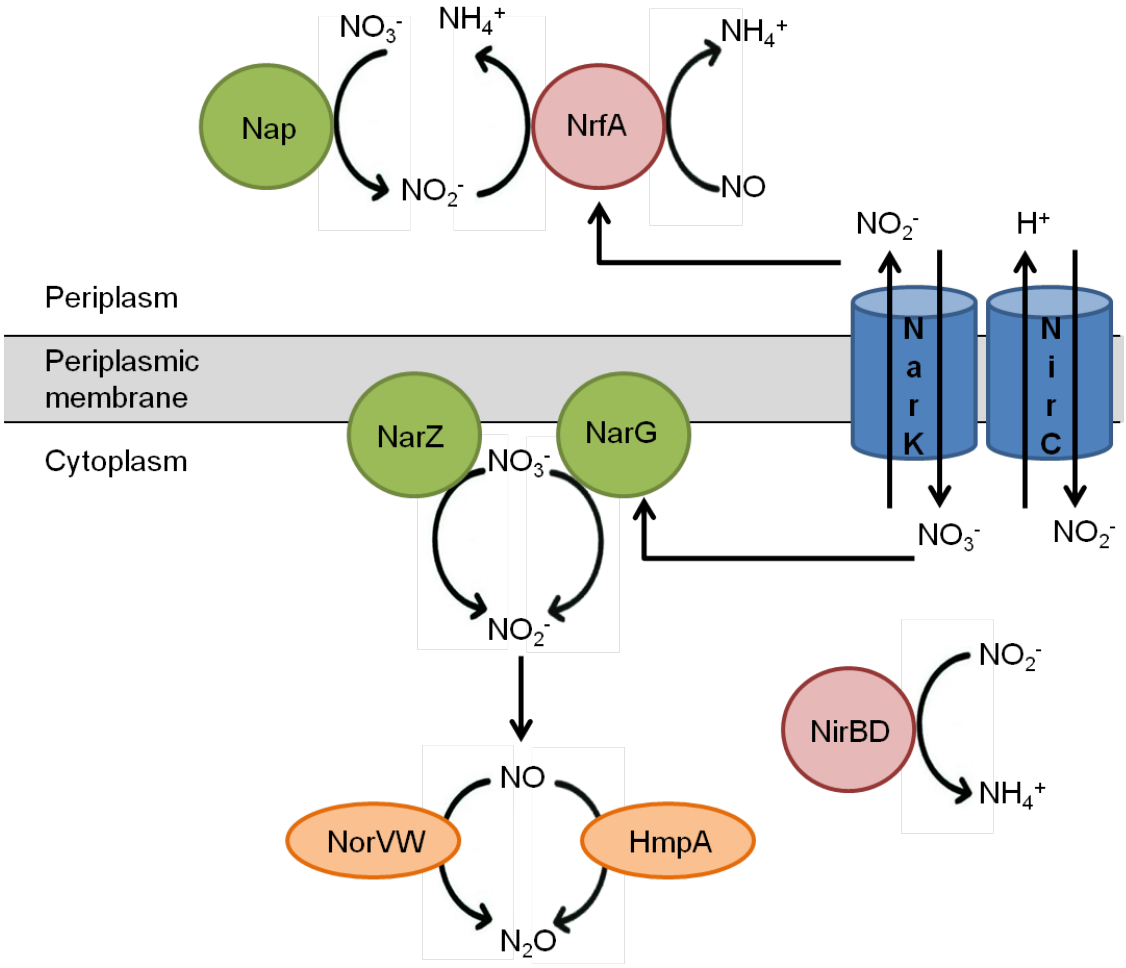

Figure 7: Location of denitrification enzymes within the bacterial cell of *E. coli* and *Salmonella*

Nap, a nitrate reductase, and NrfA, a cytochrome *c* nitrite reductase, are found in the periplasm. The antiporters NarK, NarU and NirC regulate the transport of nitrate, nitrite and protons across the periplasmic membrane. This supplies nitrate for the membrane-bound nitrate reductases NarG, NarZ or for Nap, depending on the growth conditions. Conversion of nitrite to ammonia is performed by the cytoplasmic NirBD or nitrite is converted to NO by NarG. Detoxification of NO in the periplasm is managed by NrfA, whereas the nitric oxide reductase NorVW and the flavohaemoglobin HmpA reduce NO to N_2O under anaerobic and aerobic conditions, respectively. Figure created with information provided in figures by (Rowley et al, 2012; Vine & Cole, 2011).

All in all, enzymatic conversion of NO prevents the toxicity of nitric oxide, which has a multitude of effects on the bacterial cell as described in 1.5.2. It destroys iron-sulphur clusters and leads to a deamination of nucleotides and consequently damages the bacterial DNA (Justino et al, 2007; Wink et al, 1991). Bacterial respiration is inhibited, further decreasing the chances of bacterial survival (Butler et al, 1997; Hori et al, 1996). In addition, NO causes the release of Zinc from thiol groups, thus causing changes in expression levels as the confirmation of transcription factors is altered (Tripathi et al, 2007).

The effects of oxidative and nitrosative stresses have mainly been investigated using the model organism *E. coli*. Three enzymes have been identified to be of major importance for the detoxification of nitric oxide: flavohaemoglobin (Hmp), cytochrome *c* nitrite reductase (NrfA) and flavorubredoxin (NorVW) (van Wonderen et al, 2008). Their role during NO detoxification is discussed in further detail below.

1.6.1 HmpA

HmpA, also commonly known as Hmp in *E. coli*, is a flavohaemoglobin that has the ability to perform two distinct reactions either in an oxic or anoxic environment. In the presence of oxygen, it functions as a nitric oxide dioxygenase, converting nitric oxide to nitrate (NO_3^-) in the following chemical reaction: $2\ NO + 2\ O_2 + NAD(P)H \rightarrow 2\ NO_3^- + NAD(P)^+ + H^+$ (Gardner et al, 2000; Gardner et al, 1998). Furthermore, it has been observed that the reaction is not only NADH-dependent, but is also facilitated by $Fe(II)O_2$ that allows the formation of a nitrosyldioxyl complex (Hausladen et al, 1998).

NO reductase activity of the purified HmpA protein in the absence of oxygen has also been confirmed (Kim et al, 1999; Mills et al, 2001). The NO molecules bind to HmpA and NO is converted to N_2O in the following reduction reaction: $2\ NO + 2\ H^+ + 2\ e^- \rightarrow N_2O + H_2O$ (Rowley et al, 2012). It has been proposed that although this mechanism seems plausible, the protection of cells against NO-caused damage cannot be guaranteed as the reductase levels are too low (Gardner & Gardner, 2002; Gardner, 2005).

The deletion of *hmpA* in *E. coli* increases the sensitivity of strains to NO and related nitrogen species (Hausladen et al, 1998; Membrillo-Hernández et al, 1999; Seth et al, 2012). Similar observations have been made for *S*. Typhimurium as *hmpA* deletion strains have increased aerobic sensitivity to NO, but no difference in sensitivity in comparison to the wild-type has been observed under anaerobic conditions (Crawford & Goldberg, 1998a; Gilberthorpe et al, 2007; Karlinsey et al, 2012; Mills et al, 2008).

Upon further investigation, a clearer role for HmpA during infection has been established. The increased NO sensitivity impacts on intracellular survival within macrophages, where the survival of Δ*hmpA* is significantly decreased when compared to wild-type survival (Bang et al, 2006; Gilberthorpe et al, 2007; Stevanin et al, 2002; Stevanin et al, 2007). This lead to a decreased bacterial burden in infection assays with *Nramp1*$^{+/+}$ mice (C3H/HeN), supporting the hypothesis that *hmpA* plays an important part during *Salmonella* infection, whereas no effect of *hmpA* deletion is observed for *Nramp1*$^{-/-}$ mice (C57BL/6) (Bang et al, 2006; Karlinsey et al, 2012). Nramp1 is a natural resistance-associated macrophage protein that is encoded by the *Slc11a1* gene and increases the resistance to pathogens, including *Salmonella* (Álvarez-Ordóñez et al, 2011). This effect has been attributed to the regulatory properties of Nramp1 on uptake of L-arginine for iNOS activity and the oxidative burst (Barton et al, 1995). Reduced iNOS enzyme activity levels resulting in lower NO levels have been measured in Nramp1$^{-/-}$ cells (Fritsche et al, 2003). In addition, it has been shown that Nramp1 is a divalent metal efflux pump located in the phagosomal membrane that in a pH-dependant manner limits the concentration of metals such as iron and manganese within the phagosome (Forbes & Gros, 2001). Metal limitations impair bacterial replication within the phagosome and hence limit bacterial survival.

With regards to *hmpA* expression, regulation involving the methionine synthesis regulator MetR has been shown in *E. coli* and regulation is linked to homocysteine (Hcy) (Membrillo-Hernández et al, 1998; Schell, 1993). Hcy is important for the synthesis of methionine and reacts with NO when present. Following nitrosylation by NO, Hcy is no longer available for the synthesis pathway. Up-regulation of methionine synthesis by MetR to compensate for Hcy

loss also results in increased expression of *hmpA*, which in turn provides protection against NO. As a result, the decreasing levels of NO lead to a reduced chance of Hcy nitrosylation and the methionine synthesis pathway is no longer affected by NO.

The induction of *hmpA* transcription following the exposure to NO has been observed and has been linked to repression by the NsrR regulator (Crawford & Goldberg, 1998a; Filenko et al, 2007). Furthermore, the oxygen-responsive regulator FNR represses *hmpA* expression in *E. coli* (Constantinidou et al, 2006; Overton et al, 2006a). Both studies also have highlighted that in addition to the presence of NO, nitrite and nitrate also act as triggers for increased *hmpA* expression. Studies in *Salmonella* have confirmed the regulation by NsrR, but have not confirmed regulation by MetR or iron-responsive regulator Fur (Bang et al, 2006; Karlinsey et al, 2012). Changes in oxygen concentrations have also shown to increase *hmpA* expression, supporting the role of *hmpA* in the adaptation to changing growth conditions during infection (Rowley et al, 2012).

HmpA plays an important part during NO detoxification when oxygen is present. Although the oxygen concentration during infection has been shown to be rather minimal (Eriksson et al, 2003), *in vitro* experiments using macrophages, as well as *in vivo* models using mice have repeatedly shown that HmpA plays an integral part during successful infection (Bang et al, 2006; Gilberthorpe et al, 2007; Karlinsey et al, 2012; Stevanin et al, 2002; Stevanin et al, 2007).

1.6.2 NorV

NorV has been identified as a flavorubredoxin, due to its zinc-β-lactamase-like domain at the N-terminal region, containing a non-haem di-iron site as well as its flavodoxin-like domain that contains a flavin mononucleotide (FMN) (Gomes et al, 2000). In addition, a rubredoxin-like centre has been identified at the C-terminus, but its role remains unclear. The NorV enzyme of the class of A-type flavoproteins has nitric oxide reductase activity, catalyzing the reduction of NO to N_2O, where the rubredoxin is not required for this function (Gomes et al, 2002). Furthermore, it has been shown that NorV is a tetrameric protein and creates an electron transfer chain with the gene product of the neighbouring

gene *norW* (Gomes et al, 2000). NorW is the redox partner of NorV and essential for its NO reductase activity. This activity, however, has been shown to be restricted to micro- and anaerobic environments (Gardner et al, 2002). The reaction of reducing NO to form N_2O involves the non-haem iron site and includes a nitroxyl intermediate. Even sub-micromolar concentrations of NO [< 0.5 µM NO] trigger the activity of NorVW (Gardner et al, 2003).

A change from oxic to anoxic conditions, the exposure to nitroprusside as well as *S*-nitrosoglutathione (GSNO) and the exposure to NO in aerobic and anaerobic environments have been demonstrated as triggers for increased expression of *norV* (Flatley et al, 2005; Hutchings et al, 2002; Pullan et al, 2007; Rowley et al, 2012). In connection with these changes in gene expression, NO addition has been linked to the activation of the σ^{54}-dependent regulator NorR (Gardner et al, 2003; Hutchings et al, 2002). A reduction of *norV* expression has been observed when *E. coli* cells are exposed to both H_2O_2 and NO (Baptista et al, 2012). No changes in *norR* mRNA have been detected, but electron paramagnetic resonance (EPR) analysis and enzyme activity assays reveal that H_2O_2 interferes with the binding of NO to NorR's iron centres, hindering the NO-dependent ATPase activity. It has been proposed that the oxidation of the iron centre of NorR allows a time-dependent activation of NorV, aiding in the detoxification of NO at later stages during macrophage infection. Changes in expression of *norV* are also observed during re-occurring *E. coli* urinary tract infections (Roos & Klemm, 2006). Although a putative NsrR site is located upstream of *norVW*, no NsrR regulation has been observed (Partridge et al, 2009). Similarly, no repression by FNR is indicated (Constantinidou et al, 2006).

Despite significantly increased sensitivity of a *S*. Typhimurium Δ*norV* deletion strain in anoxic conditions, no phenotypic changes in intracellular survival in macrophages using an *E. coli* deletion strain or during mice infection with *Salmonella* mutant strains are observed (Bang et al, 2006; Mills et al, 2008; Pullan et al, 2007). In agreement with enzyme activity reports, NorV does not protect the aerobic growth of *S*. Typhimurium in the presence of NO (Mills et al, 2008). These results indicate the presence of several enzymes working together to ensure the complete detoxification of NO when encountered during infection. Although NorV seems to play an important part, *in vivo* experiments

have highlighted that its nitric oxide reductase activity alone is not essential for survival of *Salmonella* during the challenging conditions of intracellular survival.

1.6.3 NrfA

nrfA encodes for a cytochrome *c* nitrite reductase (Wang & Gunsalus, 2000) and forms a $NrfA_2$-$NrfB_2$ complex that is required for enzyme activity (Bamford et al, 2002; Clarke et al, 2007). Both NrfA and NrfB are penta-haem cytochromes and are located in the periplasm, where NrfB functions as the redox partner for NrfA (Bamford et al, 2002). In *E. coli*, it has been shown that NrfA reduces nitrite to ammonia as well as reduces NO to hydroxylamine (Poock et al, 2002; Vine et al, 2011; Wang & Gunsalus, 2000). In addition, it has been found that NrfA also produces NO (Corker & Poole, 2003). This, however, has not been demonstrated for the *Salmonella* enzyme (Gilberthorpe & Poole, 2008).

Deleting *nrfA* increases the sensitivity of *E. coli* strains towards the nitrosating agent *S*-nitroso-*N*-acetyl penicillamine (SNAP) (Poock et al, 2002). In anaerobic conditions, the addition of an aqueous NO solution has similar effects on a respective *S*. Typhimurium strain, although the effect on the growth of a single mutant strain is not as big as for the Δ*norV* strain (Mills et al, 2008). However, a combination of both *norV* and *nrfA* deletions results in a significantly reduced growth and further increases the sensitivity in comparison to the phenotype of the Δ*norV* strain. When *S.* Typhimurium is exposed to endogenously produced NO in chemostat experiments, the deletion of *nrfA* does not result in any phenotypic differences compared to the parent strain (Rowley et al, 2012).

The regulation of *nrfA* is the result of a combination of several regulators. Repression of expression mediated by NsrR has been shown, which is responsive to the presence of NO (Efromovich et al, 2008). In addition, expression is also repressed by NarL, the reponse regulator of the NarQL two-component system, but is also activated by FNR and NarP (Overton et al, 2006a; Pullan et al, 2007). Such tight regulation is also supported by one study that has found a minimum of five proteins regulating the promoter of *nrfA* (Filenko et al, 2007). In summary, NrfA plays an integral part in the

detoxification of NO and is especially important under anaerobic conditions and when NorV is absent.

1.6.4 NO detoxification in other bacteria

As mentioned previously, nitric oxide detoxification is not unique to *Salmonella* or *E. coli*, but is also found in denitrifying bacteria such as *Paracoccus* and *Pseudomonas* species. Flavohaemoglobins such as Hmp have already been identified in other bacteria, including *Bacillus subtilis* and *M. tuberculosis*, where it could possibly contribute to NO tolerance as part of gut commensalism and pathogenicity, respectively (Poole & Hughes, 2000). Furthermore, evidence has been found that *Neisseria gonorrhoeae* contains an outer membrane nitrite reductase as well as a quinol-dependent NO reductase (Zumft, 2002). With regulation by NsrR and FNR, more similarities between *Salmonella*, *B*. *subtilis* and *N*. *gonorrhoeae* have become apparent (Kommineni et al, 2010; Spiro, 2007). The equine pathogen *Rhodococcus equi*, related to *M*. *tuberculosis*, displays a similar phenotype within macrophages. It is able to form and maintain *Rhodococcus*-containing vesicles in macrophages and is able to survive and multiply within this structure (von Bargen et al, 2011). Its survival relies on the successful detoxification of NO, whereas ROS have been shown to be less relevant during survival.

For the foodborne pathogen *Campylobacter*, no *norV*- or *hmpA*-homologues have been identified (Pullan et al, 2008). NrfA has been located on the *Campylobacter* genome, but its contribution to NO detoxification is only minimal (Pickford et al, 2008). However, *Campylobacter* has two globin proteins Cgb and Ctb that have been identified for their role in NO defence. The single domain globin Cgb lacks the reductase domain of a haemoglobin and has similarity to myoglobin (Elvers et al, 2004). *Cbg* expression increased with exposure to nitrosative agents and a gene deletion results in increased NO sensitivity. A possible dioxygenase or denitroxylase activity of Cgb has been proposed (Shepherd et al, 2011). Both *cgb* and *ctb* are under the regulation of the NO-sensitive transcriptional regulator NssR. A role for Ctb in *Campylobacter* respiration has been proposed, with a possible facilitation of oxygen transfer to terminal oxidases or to Cgb to support NO detoxification (Wainwright et al, 2005; Wainwright et al, 2006). Binding of oxygen or NO to Ctb has been

proposed to reduce nitrosative stress and Ctb additionally influences the expression levels of *cgb* (Smith et al, 2011). It has been observed that *Campylobacter jejuni* has a significantly higher NO resistance than *S*. Typhimurium and that by an unknown mechanism, *C. jejuni* induces the expression of iNOS in macrophages so that the levels of iNOS increase 200 fold (Iovine et al, 2008). Hence, nitrosative stress plays an integral part of innate immune defence against *Campylobacter* infections and *Campylobacter*, similar to *Salmonella*, heavily relies on NO detoxification mechanisms to be able to successfully establish an infection.

Exposure to NO is also prevalent for plant-symbiotic bacteria. The FNR-type regulator NnrR has been shown to increase the expression of *hmp*, a putative flavohaemoglobin of *Sinorhizobium meliloti*, during the exposure to NO (Meilhoc et al, 2010). Deletion of a nitric oxide reductase NorB increases the strain's sensitivity towards NO. As NO inhibits the nitrogen fixation activity of rhizobia by interfering with leghaemoglobin, detoxification e.g. by a nitric oxide reductase or a flavohaemoglobin, is additionally of importance to establish a functional symbiosis with the plant host (Meakin et al, 2007; Meilhoc et al, 2010; Tominaga et al, 2009; Wang & Ruby, 2011).

Overall, all three enzymes currently identified in *E. coli* and *Salmonella* contribute to a successful circumvention of NO poisoning, but other enzymes are likely going to be involved in the wider defence against NO. Part of this is done by a series of transcriptional regulators that allow precise tuning of gene expression in response to NO. Every organism that is likely to encounter NO as part of its lifestyle, either during infection, anaerobic metabolism or during commensalism, is most likely going to possess similar enzymes that allow it to detoxify NO in order to survive. As the enzymes share a high homology, their regulators are also highly similar.

1.7 Regulatory proteins which coordinate the NO response

There are several proteins that have been identified for their importance to provide Enterobacteriaceae with the regulatory tools to mount a successful defence against antibacterial agents such as nitric oxide. Most of these

regulators are part of a complex regulatory network of several regulators working together. Four of the most important regulators of *Salmonella* NO detoxification genes are further introduced here.

1.7.1 FNR

FNR stands for **f**umarate and **n**itrate reductase **r**egulator due to its control over the nitrate and fumarate reductase (Stock et al, 1989). It functions to adjust gene expression in the transition from aerobic to anaerobic growth by prioritizing the use of different terminal electron acceptors over one another (Overton et al, 2006a; Stock et al, 1989): In the absence of oxygen, the preferred electron acceptor, the de-repression of nitrate and nitrite reductases ensures the use of nitrate and nitrite instead of fumarate. This finely tuned regulation has been shown to primarily be the result of FNR and NarL co-regulation (Overton et al, 2006a). The FNR protein readily reacts in the presence of oxygen, converting the $[4Fe\text{-}4S]^{2+}$ cluster into a $[2Fe\text{-}2S]^{2+}$ cluster, which no longer binds DNA and hence changes the regulation of genes previously activated or repressed (Khoroshilova et al, 1997).

Microarray analysis for the comparison of gene expression of wild-type with *fnr* mutant strains has shown that a minimum of one hundred operons in *E. coli* and *S*. Typhimurium are regulated by FNR (Constantinidou et al, 2006; Fink et al, 2007; Overton et al, 2006a). Furthermore, the presence of nitrate and nitrite in addition has been shown to influence gene expression. Activation by FNR has been shown for e.g. *napA*, *nrfA*, *nirB* and *hcp*, whilst repression of *hmpA*, *ytfE* (iron sulphur repair), *ygbA* (uncharacterized gene), *yeaR-yoaG* (putative tellurite resistance) and *cyoA* (cytochrome o ubiquinol oxidase gene) has been demonstrated (the function of these genes is discussed later) (Constantinidou et al, 2006; Filenko et al, 2007; Vine & Cole, 2011).

FNR belongs to the family of FNR/CRP (cyclic adenosine monophosphate (AMP)-receptor proteins) (Spiro, 2012). Homologues of FNR and the DNA site it recognises with a hairpin loop motif (TTGAT-N_4-ATCAA) cannot only be found in *E. coli* and *Salmonella*, but also in soil-dwelling bacteria such as *P.*

denitrificans and *Bradorhizobium japonicum* as well as *P. aeruginosa* and *Rhodobacter* (Rodionov et al, 2005; Zumft, 1997; Zumft, 2005).

It contains a $[4Fe\text{-}4S]^{2+}$ cluster which reacts with oxygen and nitric oxide, resulting in a dinitrosyl-iron-cysteine complex when in contact with NO (Cruz-Ramos et al, 2002). This complex no longer allows for the dimerisation of FNR which is necessary for DNA binding and hence changes in the repression or activation of gene expression occur. This inactivation of FNR by NO has been demonstrated *in vitro* with spectrometry as well as *in vivo* using microarray experiments (Cruz-Ramos et al, 2002; Pullan et al, 2007). As a result of its reactivity with NO, FNR maintains a NO-dose dependent regulation of genes necessary for the adaptation to anaerobiosis as well as for NO detoxification. Anaerobiosis using nitrate and nitrite as terminal electron acceptors leads to the production of NO as a by-product of denitrification. Once NO is no longer detoxified by FNR-activated NrfA, the reactivity of NO with FNR occurs and lifts the repression of *hmpA* and to ensure further activation of genes involved in NO defence (Corker & Poole, 2003).

The importance of FNR for anaerobic growth has been highlighted by the poor growth that *fnr* mutant strains display (Constantinidou et al, 2006; Corker & Poole, 2003). In the absence of FNR, no endogenous NO production is observed, indicating the regulation of nitrite and nitrate reductase genes by FNR (Corker & Poole, 2003; Gilberthorpe & Poole, 2008). Genes regulated by FNR are widely similar between *S.* Typhimurium and *E. coli*, but in *Salmonella* flagella and pathogenicity genes, e.g. SPI-1 genes from the *inv* operon and *sicA*, are also under FNR-regulation (Fink et al, 2007): When *fnr* is deleted, the mutant strain is no longer motile and is hypersensitive to ROS. Furthermore, the strain cannot survive intracellular within macrophages and is highly attenuated in mice. Previously, it has been shown that FNR is essential during enteritis, but not for the development of typhoid fever, indicating that regulation in *S*. Typhimurium is different to *S*. Typhi (Rollenhagen & Bumann, 2006). The necessity of FNR for virulence is also shown for *Haemophilus influenzae*, where the *fnr* deletion strain is unable to survive in activated macrophages (Harrington et al, 2009). A proteomic study of *S*. Typhimurium during infection conditions

has further underlined the importance of FNR as it is the main regulator of adaptation (Encheva et al, 2009).

1.7.2 Fur

Fur (**f**erric **u**ptake **r**egulator) is a transcriptional regulator that has been implicated in the regulation of iron homeostasis (Kumar & Shimizu, 2011; Lucas & Lee, 2000). Fur belongs to a protein family that has been connected to the regulation of metal concentrations within the cell (Chiang & Schellhorn, 2012; Dubbs & Mongkolsuk, 2012). Fur forms homodimers in order to bind DNA (Bagg & Neilands, 1987; Touati, 2000). Each monomer contains a structural zinc molecule as well as Fe^{2+} that supports Fur activity. It has been shown that Fur binds iron when available and then binds the so-called iron box in the promoter of iron-responsive genes, which represses gene expression (de Lorenzo et al, 1987). Iron boxes with the 19 bp sequence of GATAATGATAATCATTATC have been found upstream of several genes, including the superoxide dismutase genes of the *sod* operon, the *suf* operon involved in iron homeostasis and upstream of *fur* itself (Constantinidou et al, 2006; de Lorenzo et al, 1987; Kumar & Shimizu, 2011; Outten et al, 2004; Patzer & Hantke, 1999; Zheng et al, 1999). In addition to the potential self-regulation, OxyR and SoxRS have been shown to respond to oxidative stress, e.g. by H_2O_2 (Kumar & Shimizu, 2011; Zheng et al, 1999; Zheng et al, 2001).

The iron-bound form of Fur reacts with sources of ROS as well as with nitric oxide leading to alleviation of repression by Fur (Chiang & Schellhorn, 2012; Pullan et al, 2007). The presence of hydrogen peroxide poses a risk to the cell as it reacts with Fe^{2+} to create Fe^{3+}, hydroxide ($OH^{\cdot}$) and hydroxyl radicals (OH^{-}) in the Fenton reaction (Touati, 2000). Exposure to NO also results in a reaction with Fe to form an iron-nitrosyl complex, which does not interfere with the dimerisation, but no longer exhibits DNA binding properties (D'Autréaux et al, 2002). This also corresponds to the regulation of *hmpA* in *S*. Typhimurium and has been shown for *E. coli*, although a Fur box has not been identified in the promoter region (Hernández-Urzúa et al, 2007; Justino et al, 2005; Poole & Hughes, 2000). The deletion of *fur* results in increased intracellular iron

concentrations, which lead to increased oxidative stress and DNA damage (Touati et al, 1995). For some bacteria, such as *Bacillus anthracis*, it is not possible to create a *fur* deletion strain and *fur* is deemed essential for the strain's survival (Tu et al, 2011).

Inactivation of Fur repression in response to stomach acid has been highlighted as a priming method of gene expression of *S*. Typhimurium prior to intracellular entry in the small intestine (Bourret et al, 2008; Hall & Foster, 1996). The link between the availability of iron and pH also plays a role during the acidification of the phagosome. Several studies have demonstrated increased acid sensitivity of *fur* deletion strains and attenuation in mice virulence models and intracellular survival assays in macrophages (Gilberthorpe et al, 2007; Karasova et al, 2009; Riesenberg-Wilmes et al, 1996). The regulation of the SPI-1 activator *hilA* through HilD has also been linked to Fur (Ellermeier & Slauch, 2008; Fink et al, 2007; Troxell et al, 2011).

Sensitivity against hydrogen peroxide and nitric oxide is also increased for deletion strains in comparison to wild-type strains of *E. coli* and *Staphylococcus aureus* (Mukhopadhyay et al, 2004; Richardson et al, 2006). Due to these *in vitro* and *in vivo* assay results, the use of a ∆*fur* strain has been explored as a potential vaccine strain. It has significantly reduced virulence in mice, alongside high immunogenicity necessary to stimulate the immune system to create *Salmonella*-specific antibodies (Curtiss et al, 2009).

Overall, the results stress the wide-ranging importance of Fur, not only for iron homeostasis, but also during oxidative and nitrosative stress. This link between several stresses allows *Salmonella* to mount a successful response to changes in the extra- and intracellular environment as they occur during infection.

1.7.3 NorR

In 2002, the *E. coli* protein YgaA has been identified to have a 42% protein identity with the nitric oxide reductase regulators (NorR) of *Ralstonia eutrophus* (Gardner et al, 2002). Further investigation has revealed that the adjacent genes *ygaK* and *ygbD* encode for a nitric oxide reductase and a NADH:rubredoxin oxidoreductase respectively and it has been proposed that

the genes are renamed from *ygaA-ygaK-ygbD* to *norR-norV-norW* (Gardner et al, 2002; Gomes et al, 2002). Deletion of *norR* inhibits the anaerobic endogenous production of NO, supporting the putative regulatory role of NorR on *norVW*. The presence of NorR is required for the expression of *norV* and ensures the protection of [Fe-S] clusters from NO-caused damage (Gardner et al, 2002). Other studies have further stressed the regulation of *norV* by NorR, also highlighting the impact of *norR* deletion on *E. coli* anaerobic growth in the presence of RNS (Gardner et al, 2003; Hutchings et al, 2002). The activation of *norVW* by NO is linked to NorR (Flatley et al, 2005; Gardner et al, 2003; Pullan et al, 2007). Three domains are needed for NorR regulator activity: 1) A C-terminal part is required for DNA binding; 2) a σ^{54}-dependent activator region with ATPase activity and 3) a N-terminal part containing a mononuclear iron cluster that has been linked to signalling (Gardner et al, 2003; Spiro, 2007). Homologues of the *E. coli* gene have been identified in other bacteria, such as *S*. Typhimurium, *P. aeruginosa* and *Vibrio cholerae* and a palindromic NorR-binding site (GT-(N_7)-AC) has been identified (Gardner et al, 2003; Rodionov et al, 2005). A change from aerobic to anaerobic growth results in up-regulation of *norV* as a result of endogenous NO production in *S*. Typhimurium, indicating similar regulatory mechanisms in *E. coli* and *Salmonella* (Rowley et al, 2012). Similar to FNR, oxygen reacts with the iron centre and changes the protein activity. In the case of NorR, the oxidation of the iron centre prevents NO binding and hence inhibits the ATPase activity (Baptista et al, 2012). This prevents the activation of NorV under oxidative stress responses, such as during the first stages of macrophage infection. When the nitrosative stress response is mounted, the presence of NO allows for NorV activation to detoxify NO. Intracellular survival assays with Δ*norR* and Δ*norV* deletion strains have shown that these genes are not the only players involved in NO detoxification as the deletion strains show no sign of decreased survival within macrophages (Pullan et al, 2007). A NsrR-binding site is situated between *norR* and *norV*, indicating a tight network of regulation involved in survival of nitrosative stress (Partridge et al, 2009).

1.7.4 NsrR

Using computational analysis to map predicted regulatory networks associated with the metabolism of nitrogen oxides, it has been shown that several core members of genes involved in the detoxification of NO exist both in *E. coli* and *Salmonella* (Rodionov et al, 2005). The NsrR regulator has a binding site containing an inverted repeat (AAGATGCATTTnAAATACATCTT) at multiple promoter sites of several implicated NO detoxification genes (Tucker et al, 2010). NsrR is a **n**itric oxide-**s**ensitive **r**epressor, which regulates the expression of genes such as *hmpA* (flavohaemoglobin gene), *ytfE* (iron sulphur repair), *ygbA* (uncharacterized gene), *hcp-hcr* (hydroxylamine reductase genes), *nrfA* (cytochrome c nitrite reductase gene) and *yeaR-yoaG* (putative tellurite resistance genes) (Bodenmiller & Spiro, 2006; Filenko et al, 2007; Karlinsey et al, 2012; Tucker et al, 2010). It belongs to the Rrf2 family of transcription factors due to the presence of a [Fe-S] cluster in its structure and a similarity with another family member IscR (Bodenmiller & Spiro, 2006). With regards to the iron-sulphur cluster, different compositions of clusters have been reported: all protein purifications were performed in *E. coli* using the *nsrR* gene cloned from different bacteria. NsrR, originally from *Streptomyces coelicolor,* has a [2Fe-2S] cluster and the apo form of the protein without the cluster is unable to bind to DNA (Tucker et al, 2008). Similar, NsrR from *N. gonorrhoea* contains a [2Fe-2S] cluster and its repression is relieved by the addition of NO (Isabella et al, 2009). In contrast, NsrR from *B. subtilis* contains a [4Fe-4S] cluster (Yukl et al, 2008).

All three studies have shown the reactivity of the cluster with NO. Irrespective of the nature of the cluster, the presence of NO results in the loss of DNA-binding. When present, NO replaces the iron from the iron-sulphur clusters, resulting in nitrosylated iron-complexes that cause a conformational change of the structure of the protein (Tucker et al, 2010) (Figure 8). As a result of the conformation change, the binding to DNA is impaired and the repression of gene expression is abrogated (Pullan et al, 2007). In addition, three conserved cysteines are needed for the ligation of the [Fe-S] cluster (Tucker et al, 2008).

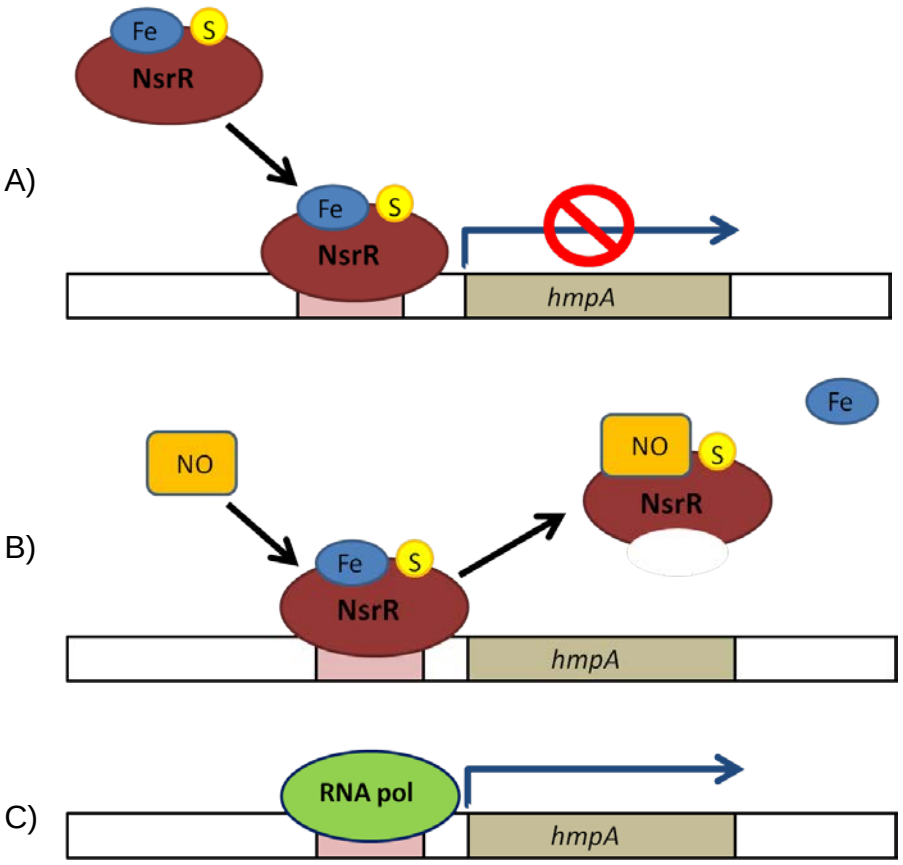

Figure 8: NsrR regulation of *hmpA*

A) The NsrR protein (maroon) containing a [Fe-S] cluster has a structure that allows binding to the DNA upstream of *hmpA*, therefore blocking the transcription initiation site (pink). No expression of *hmpA* takes place.

B) The presence of NO results in the removal of iron from the [Fe-S] cluster, therefore causing a conformational change. Binding to the DNA is no longer possible and NsrR dissipates.

C) The transcription initiation site becomes available for binding by the RNA polymerase (green) and transcription of *hmpA* takes place.

Binding of NsrR to the DNA is believed to occur after dimerisation of NsrR, where each monomer binds to one of the two halves of the binding site. This binding blocks the transcription site where binding of the RNA polymerase would take place.

Further experiments have highlighted the largely conserved structure of the NsrR-binding site for *E. coli* and *S*. Typhimurium (Bodenmiller & Spiro, 2006; Karlinsey et al, 2012; Lin et al, 2007; Rodionov et al, 2005) (Table 1). In addition, previously predicted NsrR-regulated genes *hmpA*, *ygbA*, *ytfE* and *hcp-hcr* are shown to be under NsrR repression for both *E. coli* and *Salmonella* (Bodenmiller & Spiro, 2006; Filenko et al, 2007; Karlinsey et al, 2012; Pullan et al, 2007). Furthermore, the *yeaR-yoaG* operon is NsrR-repressed for both organisms (Filenko et al, 2007; Karlinsey et al, 2012; Lin et al, 2007).

The deletion of *nsrR* increases the resistance of *Salmonella* towards GSNO and NO as a result of de-repressed NO detoxification genes such as *hmpA* (Gilberthorpe et al, 2007; Karlinsey et al, 2012). However, the survival in RAW264.7 macrophages of the deletion strain is decreased in comparison to the parent strain (Gilberthorpe et al, 2007). This is explained with the necessity to ensure the tight regulation of *hmpA* during infection. During the exposure to peroxide and superoxide that are creating oxidative stress, the deletion of *nsrR* increases the strain's sensitivity.
Overall, this highlights the importance of NsrR for *Salmonella*'s survival during infection and shows the need for regulation of NO detoxification genes. In *N. gonorrhoea* the deletion strain has an increased resistance towards nitrite shock, indicating a similar involvement of NsrR for the pathogenicity of *Neisseria* (Overton et al, 2006b).

All in all, NsrR is an important regulator of genes that in part has been shown to help with NO detoxification. Its responsiveness to NO ensures that NO detoxification genes are only expressed in the presence of NO rather than continuously, which is an energy-efficient way of regulating gene expression.

Table 1: NsrR-regulated genes identified in *E. coli* and *S.* Typhimurium
(*) *S.* Typhimurium-specific gene

Gene(s):	References:	
	E. coli:	*S. Typhimurium*:
hcp-hcr (hydroxylamine reductase; NADPH oxidoreductase)	(Filenko et al, 2007; Pullan et al, 2007; Rodionov et al, 2005)	(Karlinsey et al, 2012; Rodionov et al, 2005)
hmpA (flavohaemoglobin)	(Bodenmiller & Spiro, 2006; Pullan et al, 2007; Rodionov et al, 2005)	(Bang et al, 2006; Gilberthorpe et al, 2007; Karlinsey et al, 2012; Rodionov et al, 2005)
nrfA (cytochrome c nitrite reductase)	(Filenko et al, 2007)	
*STM1808** (putative tellurite resistance)		(Karlinsey et al, 2012; Rodionov et al, 2005)
tehAB (tellurite resistance)	(Bodenmiller & Spiro, 2006)	(Rodionov et al, 2005)
yeaR-yoaG (putative tellurite resistance)	(Filenko et al, 2007; Lin et al, 2007; Squire et al, 2009)	(Karlinsey et al, 2012)
ygbA (uncharacterized)	(Bodenmiller & Spiro, 2006; Pullan et al, 2007; Rodionov et al, 2005)	(Gilberthorpe et al, 2007; Karlinsey et al, 2012)
ytfE (iron sulphur repair)	(Bodenmiller & Spiro, 2006; Pullan et al, 2007; Rodionov et al, 2005)	(Gilberthorpe et al, 2007; Karlinsey et al, 2012; Rodionov et al, 2005)

1.8 Summary and context

Salmonella ssp. have evolved into a range of serovars able to cause disease in a range of animal species, including humans. Its ability to maintain a reservoir in a range of different species enables wide-spread occurrence of infections around the world. Salmonellosis manifests itself in one of two different types of illness: gastroenteritis and enteric fever. Both diseases potentially have fatal outcomes, especially in immunocompromised individuals, the elderly, young children and babies. For example, the combination of Malaria infection with *Salmonella*-caused gastroenteritis has detrimental effects in children. The emergence of strains increasingly resistant against a range of antibiotics has decreased the chances of successful treatment in the case of infection. Currently available vaccines, however, only offer a limited choice of prevention since the storage conditions, patient range and the course of vaccination are not ideal. The hot climate, the age of patients most at risk of dying from Salmonellosis as well as accessibility of healthcare are notable obstacles in the countries most commonly affected. Therefore several projects have looked at increasing the sanitary conditions and at providing guidance on food preparation as a more effective means of preventing infection.

Ingestion of food and water contaminated with *Salmonella* is one of the most common sources for *Salmonella* infections. Within the stomach, the gastric juice triggers the acid-tolerance response that allows its survival by modulating gene expression. It also allows priming of the expression of genes that becomes important for *Salmonella* infection later on in the intestine. Upon reaching the small intestine, *Salmonella* induces its uptake into intestinal epithelial cells and crosses the basolateral layer via the passage through M cells. Uptake into epithelial cells involves the induction of membrane ruffling. This is mediated in *S*. Typhimurium by the expression of SPI-1, where genes encode for a range of proteins that allow the formation of a needle complex as well as effector proteins that interact with the cytoskeleton of the host cell. Once inside the cell, *Salmonella* hijacks the cell's nutrients for its own metabolism. It also is a means of escaping the recognition by the immune system.

M cells filter the gut lumen for foreign antigens. Once in contact with *Salmonella*, they take up the bacteria and present them to macrophages on the

other side of the intestinal lumen. Macrophages are specialised to take up bacteria via phagocytosis. The phagosome containing the bacteria later fuses with the lysosome that contains antimicrobial peptides, ROS and RNS for the elimination of pathogens. Once the bacteria have been killed, macrophages are then able to present antigens to B and T cells. Those cells, belonging to the adaptive immune system, allow for the mounting of a complete immune response that includes antibody production for early recognition and defence against future infections.

Within the macrophage a range of reactive oxygen and nitrogen species as well as intermediates are released into the phagosome. These radicals react with each other as well as with metals, releasing further radicals. The structure of proteins is also further at risk to NO binding. Elimination of enzyme activity or of binding properties significantly impacts on the bacterial cell metabolism and inhibition of bacterial respiration has also been reported. All in all, these defence mechanisms are very effective in preventing infections.

However, *Salmonella* has evolved various proteins that allow its survival inside macrophages. It has enzymes that allow for the conversion of ROS and RNS into less toxic chemical compounds. These enzymes include i) the flavohaemoglobin HmpA that converts NO into nitrate; ii) the flavorubredoxin and nitric oxide reductase NorV that reduces NO to N_2O and iii) the cytochrome *c* nitrite reductase NrfA that converts NO into ammonia. These enzymes have specific oxygen requirements and are not all expressed at the same time. For aerobic conditions, HmpA has been shown to be most important, but it also contributes to intracellular survival and mice virulence assays. The activity of NrfA has been shown to contribute to survival in the stomach. In the absence of oxygen, NorV has been highlighted to be of most importance for NO detoxification. Deletion of all three genes significantly reduces the growth in the presence of NO in *in vitro* anaerobic experiments. Aerobically the effect is less distinct and *in vivo* experiments are yet to prove that these are the only proteins providing protection against NO under the complex environmental conditions of infection.

There are a number of regulators that support the expression of the NO detoxification genes. FNR mainly regulates the use of fumarate and nitrate as electron acceptor sources during anaerobiosis, but has shown changes in activity and gene repression after exposure to NO. The main task of Fur is to regulate the iron uptake of the cells. Due to the reactivity of NO with protein-bound iron, its regulation also responds to the presence of NO. Similar to FNR and Fur, NsrR contains an iron-sulphur cluster that is nitrosated by NO. The conformational changes result in a de-repression of genes such as *hmpA*, which is then transcribed to yield an effective NO defence. Unlike the other three regulator described, NorR seems to provide an exclusive regulation of *norVW* only, in comparison to more than 30 operons under the regulation of Fur.

Overall, the interplay between the human immune system and *Salmonella* still has various unanswered questions and it remains unknown, which other genes may be important for the detoxification of NO for *Salmonella* pathogenesis. Novel mechanisms will not only further enhance our understanding of *Salmonella* pathogenesis, but could also be suitable targets for future drug or vaccine development.

This project further explores which other genes support *Salmonella* during detoxification of nitric oxide by comparing two datasets of gene expression changes obtained during exposure to exogenous and endogenous NO, respectively. Following on, the gene set identified is tested for sensitivity of mutant strains towards anaerobic growth in the presence of NO. In addition, tellurite sensitivity of the deletion strains of genes *STM1808*, *yeaR*, *tehA* and *tehB* is examined to elucidate a possible link between tellurite and NO sensitivity. Furthermore, intracellular survival in activated and non-activated murine macrophages is investigated as a step towards a more complex environment mimicking the route of infection of *Salmonella*. The phenotypes are compared and contrasted in order to provide a clearer picture of additional nitric oxide detoxification mechanisms in *S*. Typhimurium. Possible routes of future work are shown that could help to identify suitable to support antibiotic or vaccine development.

2 Materials & Methods

2.1 Strains & culture conditions

2.1.1 Strains & plasmids

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

2.1.2 Complex media

The following complex media are used in this study:

- Luria-Bertani (LB) broth:

  10 g Tryptone, 5 g Yeast Extract, 10 g NaCl per 1 L distilled H_2O (dH_2O); pH adjusted to pH 7.5

- Lennox broth:

  10 g Tryptone, 5 g Yeast Extract, 5 g NaCl per 1 L dH_2O

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

  8 g Tryptone, 1g Yeast Extract, 5 g NaCl, 1.5% (w/v) agar per 1 L dH_2O; addition of 21 mL of 40% (w/v) glucose (F/S), 25 mL of 2.5% (w/v) alizarin yellow G and 3.3 mL of 2% (w/v) aniline blue (F/S) after autoclaving.

Sterilization of media is achieved by autoclaving at 121°C for 15 min. The supplementation of media with Kanamycin [75 µg x mL^{-1}], Chloramphenicol [10 µg x mL^{-1}], Ampicillin [100 µg x mL^{-1}] or Tetracycline [5 µg x mL^{-1}] is performed, when appropriate.

2.1.3 Overnight cultures

Overnight cultures are prepared using LB broth, supplemented with appropriate antibiotics. The bacterial cultures are incubated overnight at 37°C with shaking at 200 rpm, unless otherwise stated.

2.1.4 Minimal media

The chemical composition of the minimal media used is outlined in Table 3. MGN is a minimal medium containing glycerol and nitrate. MGM indicates minimal glucose content.

Table 2: Strains and plasmids used in this study:

Strain	**Genotype**	**Source**
SL1344	*Salmonella enterica* serovar Typhimurium 4/74 *hisG rpsL*	(Hoiseth & Stocker, 1981)
SL1344 pSTM1808$_{prom}$	*S.* Typhimurium pMP220 STM1808$_{prom}$ (Amp^R)	This study
Δ*cstA*	SL1344 Δ*cstA*::cat	This study
Δ*cstA* p*cstA*	SL1344 Δ*cstA*::cat, pBAD *cstA* (Amp^R)	This study
Δ*cydB*	SL1344 Δ*cydB*::kan	This study
Δ*cydB* p*cydB*	SL1344 Δ*cydB*::kan, pBAD *cydB* (Amp^R)	This study
Δ*hcr*	SL1344 Δ*hcr*::kan	This study
Δ*hcr* p*hcr*	SL1344 Δ*hcr*::kan, pBAD *hcr* (Amp^R)	This study
Δ*hmpA*	SL1344 Δ*hmpA*::kan	(Mills et al, 2008)
Δ*hmpA* p*hmpA*	SL1344 Δ*hmpA*::kan, pBAD *hmpA* (Amp^R)	This study
Δ*napABCD*	SL1344 Δ*napABCD*::kan	(Appia-Ayme et al, 2011)
Δ*narGHIJ*	SL1344 Δ*narGHIJ*::kan	(Rowley et al, 2012)
Δ*narVWYZ*	SL1344 Δ*narVWYZ*::kan	(Appia-Ayme et al, 2011)
Δ*napABCD* Δ*narGHIJ* Δ*narVWYZ*	SL1344 Δ*napABCD*::pCP20, Δ*narGHIJ*::cat, Δ*narVWYZ*::kan	(Appia-Ayme et al, 2011)
Δ*nirB*	SL1344 Δ*nirB*::kan	(Rowley et al, 2012)
Δ*norV*	SL1344 Δ*norV*::kan	(Mills et al, 2008)
Δ*nrfA*	SL1344 Δ*nrfA::*kan	(Mills et al, 2008)
Δ*nsrR*	SL1344 Δ *nsrR*::kan	This study
Δ*STM1273*	SL1344 Δ*STM1273*::kan	This study
Δ*STM1808*	SL1344 Δ*STM1808*::cat	This study
Δ*STM1808* p*STM1808*	SL1344 Δ*STM1808*::cat*,* pBAD STM1808 (Amp^R)	This study
Δ*STM1808* Δ*tehB* Δ*yeaR*	SL1344 Δ*STM1808*::cat, Δ*tehB::pCP20,* Δ*yeaR*::kan	This study
Δ*tehA*	SL1344 Δ*tehA*::cat	This study
Δ*tehB*	SL1344 Δ*tehB*::kan	This study
Δ*tehB* p*tehB*	SL1344 Δ*tehB*::kan, pBAD *tehB* (Amp^R)	This study
Δ*tehB* Δ*yeaR*	SL1344 Δ*tehB*::pCP20, Δ*yeaR*::kan	This study
Δ*yeaR*	SL1344 Δ*yeaR*::kan	This study
Δ*yeaR* p*yeaR*	SL1344 Δ*yeaR*::kan, pBAD *yeaR* (Amp^R)	This study
Δ*ygbA*	SL1344 Δ*ygbA*::cat	This study
Δ*ygbA* p*ygbA*	SL1344 Δ*ygbA*::cat, pBAD *ygbA* (Amp^R)	This study
Δ*ytfE*	SL1344 Δ*ytfE*::kan	This study
Δ*ytfE* p*ytfE*	SL1344 Δ*ytfE*::kan, pBAD *ytfE* (Amp^R)	This study
Plasmid	**Genotype**	**Source**
pBAD/*Myc*-His A	C-Terminal 6xHis Tags, Amp^R	Invitrogen™
pCP20	Temperature-sensitive replication and thermal induction of FLP synthesis, Amp^R, Cat^R	(Cherepanov & Wackernagel, 1995)
pKD3	Amp^R, pANT-Sγ derivative, Cat^R	(Datsenko & Wanner, 2000)
pKD4	Amp^R, pANT-Sγ derivative containing a FRT-flanked Kan^R	(Datsenko & Wanner, 2000)
pKD46	Amp^R, pINT-ts derivative containing *araC*-P$_{araB}$ and γ, β, exo genes	(Datsenko & Wanner, 2000)
pMP220	Wide host-range promoterless-*lacZ* probe vector (Tet^R), low-copy number	(Zaat et al, 1987)

The basic composition of MGM and MGN has been described previously (Pope & Cole, 1982). Since the wild-type strain used is a histidine auxotroph, a supplementation with casamino acids is required for growth in minimal media.

2.2 General laboratory techniques

2.2.1 Polymerase chain reaction (PCR)

The following table lists the PCR programs used during this project. In general, an elongation time of approximately 1 min per 1 kb product is used. All the reaction mixtures and programmes can be found in Table 4 and Table 5, respectively.

2.2.2 PCR product purification

The purification of PCR products is performed using a QIAquick PCR Purification Kit (QIAGEN) and following the manufacturer's instructions. Briefly, the PCR sample is mixed with five volumes of binding buffer (PB) to enable binding of the DNA to a centrifuge column. The sample is applied to a centrifuge column and centrifuged at 13,000 rpm and for 1 min. The eluate is discarded and the column is washed with 0.75 mL of PE buffer. After removal of the eluate, an additional centrifugation step ensures the sufficient removal of ethanol from the buffer. Elution of the DNA is done using 50 µL of dH_2O (Sigma-Aldrich), leaving the column to stand for 1 min before centrifugation. Quality of the purification is routinely checked using gel electrophoresis (see section 2.2.4).

2.2.3 Plasmid purification

The purification of plasmids has been performed using a QIAprep Spin Miniprep Kit (QIAGEN) and following the manufacturer's instructions. Briefly, 10 mL of a LB overnight culture with the appropriate antibiotic are centrifuged at 13,000 rpm for 3 min at room temperature. The supernatant is removed and the bacterial pellet resuspended in 500 µL of P1 buffer. Next, 500 µL of the lysis buffer (P2) are added and mixed by inverting the tube several times.

Table 3: Chemical composition of minimal media used in this study

All the media are autoclaved at 121°C for 15 min. Casamino acids and glucose are prepared separately, filter-sterilised using a 20 µm filter and added just before use. Glycerol and sodium nitrate are prepared separately, autoclaved and also added just before use. * For aerobic growth, 0.01% (w/v) casamino acids are added. To enhance anaerobic growth, the amount of casamino acids is increased to 0.05% (w/v).

Common name:	**chemical formula:**	**MGN:**	**MGM:**
potassium dihydrogen phosphate	KH_2PO_4	33 mM	33 mM
dipotassium phosphate	K_2HPO_4	30 mM	60 mM
ammonium sulphate	$(NH_4)_2SO_4$	8 mM	7.5 mM
tri-sodium citrate	$Na_3C_6H_5O_7$	2 mM	2 mM
magnesium sulphate	$MgSO_4$ x 7 H_2O	200 µM	200 µM
ammonium hepta-molybdate	$(NH_4)_6Mo_7O_{24}$	1 µM	1 µM
sodium selenate	Na_2SeO_4	1 µM	0.8 µM
magnesium chloride	$MgCl_2$ x 4 H_2O	400 µM	450 µM
manganese chloride	$MnCl_2$ x 4 H_2O	50 µM	50 µM
calcium chloride	$CaCl_2$	9 µM	11.5 µM
iron chloride	$Fe(II)Cl_2$ x 4 H_2O	160 µM	10.5 µM
sodium nitrate	$NaNO_3$	22 mM	-
glycerol	$C_3H_5(OH)_3$	5 mM	-
glucose	$C_6H_{12}O_6$	-	55 mM
casamino acids		0.1 mg x L^{-1}	0.01%/ 0.05%*

Table 4: Components of the PCR reaction

Reagent:	**Volume [µL]:**
BIOMIX (Bioline) (2x)	25
5' - 3' primer [20 µM]	1
3' - 5' primer [20 µM]	1
DNA	4
dH_2O (Sigma Aldrich)	19
Total volume:	50

Table 5: Details of the PCR programs used

Use of the program:	**Analysis of mutagenesis:**		**Knockout construction:**	
Description	temp [°C]:	t [min]:	temp [°C]:	t [min]:
1) Initial denaturation	95	2	94	2
2) Denaturation	95	2	94	0.25
3) Annealing	45	1	55	0.5
4) Elongation	72	2	72	1
5) Repeat of steps 2) to 4)	30x		26x	
6) Final elongation	72	10	72	7

By adding 700 µL of buffer N3 and mixing the solution by inverting, the pH of the mixture is neutralized. The solution is centrifuged at 13,000 rpm for 10 min and the supernatant is applied to a centrifuge column. The following centrifugations are all performed at 13,000 rpm for 1 min unless otherwise stated. For binding of the plasmid DNA to the column, the column with the supernatant is centrifuged. The flow-through is discarded and the column washed with 500 µL PB buffer by centrifuging the column again. The flow-through is again discarded and another washing step using 750 µL PE buffer followed by another centrifugation step. To eliminate residual ethanol from the PE buffer, the column is centrifuged again after the removal of the flow-through. The DNA is then eluted using 50 µL of dH_2O, leaving the water on the column for 1 min before a final centrifugation step. Using a NanoDrop2000 (ThermoScientific), the quantity of the plasmid is assessed. Alternatively, quality of the purified plasmid is assessed using gel electrophoresis (see section 2.2.4).

2.2.4 Gel electrophoresis

The quantity and quality of PCR products and plasmids are analyzed using gel electrophoresis. For loading, either 5 µL of PCR product or a mixture of 1 µL purified product or plasmid mixed with 1 µL loading dye and 4 µL dH_2O is used for analysis on a 1% (w/v) agarose gel containing 0.004% (v/v) Ethidium Bromide. The gel is run at 100 V for 45 minutes. For size comparison, a 1 kilobase (kb) Hyperladder (Bioline) is run as a marker. Imaging of the gel is done under exposure to UV light.

2.3 Generation of constructs for gene knockouts

Primers have been designed to fulfil the following criteria: Firstly, to contain a homology region with the gene of interest (indicated as P1 and P2 in Figure 9A respectively) and secondly, to contain a priming sequence of the antibiotic resistance cassette (indicated as H1 and H2 respectively). A list of primers used in this study can be found in Table 6. The primers are then used in a PCR performed by the BIO-RAD DNAEngine® to amplify an antibiotic gene cassette either from the plasmid pKD3 (Cat^R) or pKD4 (Kan^R) with homologous tails specific for the gene of interest. A detailed description of the different steps of the PCR is given in 2.2.1 and Table 5.

Table 6: List of primers used for the generation of gene knockout constructs

Gene:	Forward primer (5' to 3'):	Reverse primer (3' - 5'):
cstA	AATGTAACATCTCTCTGGAAC ACCCAAACGGACAACAACTG TGTAGGCTGGAGCTGCTT	CCCTCTCCTTATTCTGGAGAG GGCTATTGATGTAAAAAGACA TATGAATATCCTCCTTAG
cydB	CGTGACTTCTCAGCCGGCAC GCTAAGACAGGAGTCGTCAA GTGTAGGCTGGAGCTGCTTC	CCCAGAATCCATGCGAAATA CCACATTTTAAGCTCCTTACC ATATGAATATCCTCCTTAG
hcr	AAGAAGACATGAAGCAATTG CTGAGCGCGTAAGGAGGTCA GTGTAGGCTGGAGCTGCTTC	GCGCTGACGCTTACCGGGCC TACGATGGAACGTTTACCGA CATATGAATATCCTCCTTAG
nirB	CGTTAAGGTAGGCGGTAATA GAAAAGAAATCGAGGCAAAA GTGTAGGCTGGAGCTGCTTC	GGCAGGATGTCATCGATTTT GCAGATGTTTTGCCACTGAC CATATGAATATCCTCCTTAG
nsrR	TAAATGTATTTTTCCCGTTTTC CCTTTTCCTGAGGTTGATGTG TAGGCTGGAGCTGCTTC	CATTGAGGTTCCTCCATTGTC ATCTCTAATGAAGTTTACTCA TATGAATATCCTCCTTAG
STM1273	CTTATGATTGGGTATTAAAAA CCAGAAGAGTCTTATAATCGT GTAGGCTGGAGCTGCTTC	ATCTATCCCATGTCGAATTCA TGGTCTTTTTTCAGCCGCGCA TATGAATATCCTCCTTAG
STM1808	TTAAATACATCTTTTAATCACC ACATCAGGGAGATGTCTTGT GTAGGCTGGAGCTGCTTC	CTGGGCGGGACGCCGCCCA GTGGTGACTGGTTTACCGAT GCATATGAATATCCTCCTTAG
tehA	CGTAATCACAAGCAGAGCGA TCGCGTGCTCAATCTGCCTG GTGTAGGCTGGAGCTGCTTC	CGAACGGTCATTTTTTTCCTC CGTTTTCAACAGTGCAGCGC ATATGAATATCCTCCTTAG
tehB	CGCACTGAGCGCGCTGCACT GTTGAAAACGGAGGAAAAAA GTGTAGGCTGGAGCTGCTTC	AAAATGCCGAACGCAGCGAG ACTTACAGAACTTGCATCGCC ATATGAATATCCTCCTTAG
yeaR	TGAGATTATCGCTGAGTAACC TGCGTGAAGAGGGAAGCAAG TGTAGGCTGGAGCTGCTTC	TTATTCGTGACCGTGACGGT ATAAGTAGCTTTTCCCATTAC ATATGAATATCCTCCTTAG
ygbA	GGTGTAAAATAAATACACCTT AATGTTCGGAGAGAGCACGG TGTAGGCTGGAGCTGCTTC	GCCTGGAGATGATGCGCGCC GTAGGCCTGGCGCTTCCCCT CATATGAATATCCTCCTTAG
ytfE	TTAAAATACAACTTATATTATT GCAAATGAGGTAACGGCTGT GTAGGCTGGAGCTGCTTC	CCGTTTACAGCCGCCATCCG GCAAAATGCCCGGCTGGCGA CATATGAATATCCTCCTTAG

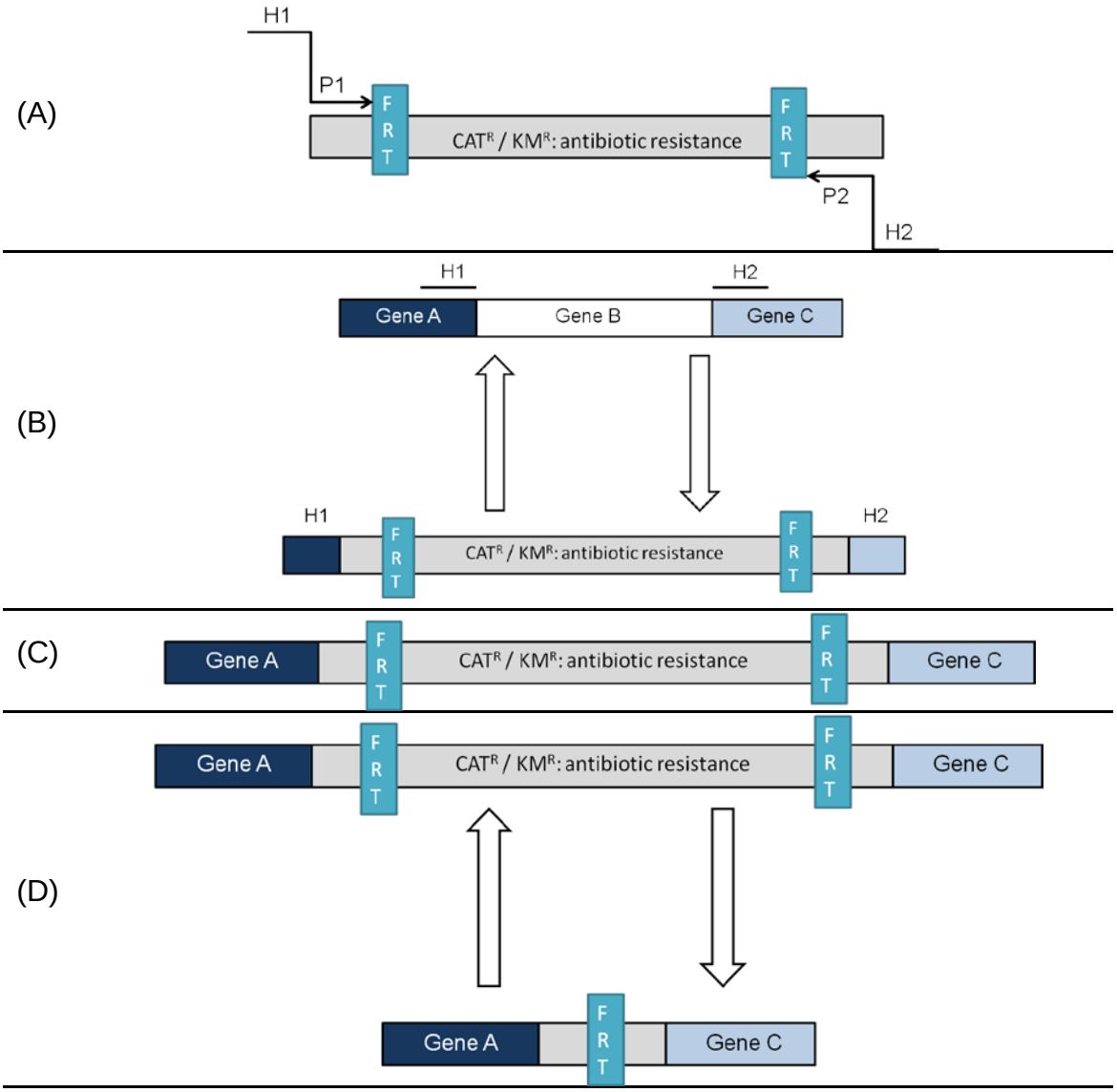

Figure 9: Creating gene knockouts

(A) Primer design and amplification: Primers are designed to contain a homology region H1 or H2 corresponding to an outside region of the gene of interest (Gene B) and a sequence of nucleotides (P1 or P2) corresponding to regions of the antibiotic resistance gene cassette of pKD3 or pKD4 outside of the FRT regions. These primers are used to create gene knockout constructs by amplifying the antibiotic resistance gene cassettes containing short sequences homologous to Gene B.
(B) Homologous recombination: The recombinase enzyme of the phage λ (γ, β, exo) (Datsenko & Wanner, 2000), encoded on the inducible plasmid pKD46, recognizes the homologous elements of the gene knockout construct and Gene B. The exchange of Gene B with the gene knockout construct takes place.
(C) Result of the recombination event: The antibiotic resistance gene containing the FRT regions has replaced Gene B without affecting the neighbouring genes A or C.
(D) Removal of the antibiotic resistance gene: The Flp recombinase of the pCP20 plasmid removes the antibiotic resistance genes due to the presence of specific recognition targets (FRT). The antibiotic resistance gene is excised and the chromosomal DNA ends joined together.

The resulting product is then purified using a QIAGEN PCR purification kit as described in 2.2.2. The purified product should have a concentration ranging from 20 to 60 ng x μL^{-1}, which is analyzed using a NanoDrop2000 (Thermo Scientific).

2.4 *De novo* mutagenesis

The wild-type strain SL1344 containing the temperature-sensitive plasmid pKD46 is grown overnight at 30°C in LB broth supplemented with Ampicillin and grown as outlined 2.1.3. 50 mL of Lennox broth containing Ampicillin [100 µg x mL^{-1}] and 1mM L-arabinose are inoculated with 1% of the overnight culture. The culture is incubated at 30°C until the OD_{600nm} reaches a value between 0.48 and 0.6. The liquid is transferred into a centrifuge tube and the cells are pelleted by centrifugation for 10 min at 4000 rpm and at 4°C. The cell pellet is washed and re-suspended three times using ice-cold 10% (v/v) glycerol. After the final washing step, 10% (v/v) glycerol is used to re-suspend the pellet a final time. 100 µL of cells are mixed with 10 µL of pKD4 or pKD3 DNA generated as described in 2.2.3. The cell-DNA mixture is then transferred into an electroporation cuvette with a gap of 0.2 cm. Electroporation is performed using the BIO-RAD MicroPulser™ electroporator at a voltage of 2.5kV for a time of 5 ms.

After electroporation, 1 mL of LB is added to the cells, the mixture is transferred to an Eppendorf tube and incubated at 37°C for 2 hours to allow the recovery of the cells and the recombination to occur. The so-called Red system of the λ phage with its recombination enzyme is found on the pKD46 plasmid and enables the recognition of the homology regions of the gene of interest (Gene B in Figure 9) as well as of the gene knockout construct (Figure 9B) (Datsenko & Wanner, 2000). It enables the exchange of those to regions, resulting in the replacement of the gene of interest Gene B with the antibiotic gene resistance cassette (Figure 9C). 100 µL of the cell suspension are plated onto LB Agar plates supplemented with Kanamycin [75 µg x mL^{-1}] (for pKD4 constructs) or Chloramphenicol [10 µg x mL^{-1}] (for pKD3 constructs) respectively. The plates are incubated overnight at 37°C.

2.5 Verification of mutagenesis

One colony is re-suspended in 200 µL of 1x PBS in a centrifuge tube. After boiling the bacterial suspension for 5 min at 100°C, it is centrifuged for 3 min at 5000 rpm. The supernatant containing the DNA is transferred into a fresh centrifuge tube and kept on ice. This DNA is then used for a PCR reaction as described in 2.2.1. The DNA of the SL1344 wild-type strain is used as a control. The products of the PCR reactions are analyzed on a 1% agarose gel, run at 110V for 45 min. A 1kb hyperladder (Bioline) is used as a size marker. Table 7 shows a list of the primers used for the verification of the gene deletions.

2.6 P22 transduction

In order to prevent further recombination events taking place once the gene deletion is accomplished in a SL1344 pKD46 strain, a transduction using the bacteriophage P22 is performed. One colony of the deletion strain in the pKD46 background is used to inoculate 10 mL of LB and grown overnight. Then, 10 mL LB are inoculated with 1% (v/v) the next day and grown aerobically at 37°C for one hour. 10 µL of P22 lysate are added and growth is continued for 6 hours. 500 µL $CHCl_3$ are added, carefully mixed and left to rest in the fridge overnight. The mixture is centrifuged for 15 min in an ice-cold centrifuge at full speed and the supernatant is transferred into a clean tube. 10 µL are mixed with 100 µL of SL1344 and incubated at 37 °C for 45 min. The cells are streaked onto LB plates supplemented with the appropriate antibiotic. After overnight growth at 37°C, six colonies are picked and streaked onto Green plates. Grown overnight at 37°C, light green colonies are picked, indicating non-lysogenic strains and again streaked onto Green plates. Following this selection, the presence of the antibiotic gene cassette instead of the gene of interest is analyzed following the protocol in 2.5.

Table 7: List of primers used for the analysis and verification of gene deletion mutant strains

Gene:	Forward primer (5' - 3'):	Reverse primer (3' - 5'):
cstA	CACCCAAACGGACAACAACT	GGGCTATTGATGTAAAAAGA
cydB	GCTGACCGTGGGCGATCTGC	TTAGCTTTCTTCTTGACCGG
hcr	CACCGGGCCGACCGCGCCGG	AAGTTTAGTTGAAGATGACG
hmpA	TTCACATAAAGGAAGCACGT	TTTCAGAGGATTTGTTGCAA
nirB	GGTCTAAAAACCCCTCATTT	CTGATCGCTATGGTAAGGAC
nsrR	TTTCCTTCCCCGAACTGA	CCGGCTCGGGATAGGATT
STM1273	GCGCTGTTTATTTACATCAG	CGAGTGGTTGAGTTTATAACC
STM1808	CCACATCAGGGAGATGTCTT	TGGTGACTGGTTTACCGATG
tehA	TACTTTATAAATTAAACAAA	TCTCAGTAAAGTAATTTTCG
tehB	GTTGAAAACGGAGGAAAAAA	ACTTACAGAACTTGCATCGC
yeaR	GTAACCAATAAATGGTAT	TCAGGTACCAGCAACGTC
ygbA	TAATGTTCGGAGAGAGCACG	GTAGGCCTGGCGCTTCCCCT
ytfE	TCATACTCGCTTAAATTA	GGTGGCGCTGGCGCTTAC

2.7 Removal of the antibiotic resistance gene cassette

The removal of the antibiotic resistance gene cassette allows generating deletion strains where both original deletion strains have the same antibiotic resistance gene cassette or where antibiotic resistance might interfere with the experiments. It is necessary to generate electro-competent cells of the strain of interest using the method outlined in 2.4. 100 µL of electro-competent cells are then transformed using 5 µL of pCP20, a plasmid containing the Flp recombinase gene. Recovery of the cells and recombination are ensured by incubating the cells statically at 30°C for three hours. During this time, the expression of pCP20 leads to the production of Flp recombinase, an enzyme originally from *Saccharomyces cerevisiae* (Cherepanov & Wackernagel, 1995). Flp recombinase recognizes specific sequences, so-called Flp recombinase targets (FRT). Recognition of FRT consequently leads to the cutting of the DNA at these target sites. As FRT are strategically placed in the beginning and the end of the antibiotic resistance cassette genes, this process leads to the removal of the cassette (Figure 9D). Due to the temperature-sensitive nature of pCP20, the cells are spread onto LB plates containing Ampicillin (Amp) and overnight incubation occurs at 30°C. After a second selection round on LB Amp plates, the strain is grown in 10 mL LB at 45 °C to remove the pCP20 plasmid. Successful removal is monitored by plating bacteria onto LB and LB Amp plates

as well as PCR analysis to confirm the removal of the antibiotic resistance gene.

2.8 Complementation studies

In order to verify the phenotypes of the deletion strains, constructs of protein expression vector pBAD/Myc-His (Invitrogen) containing the gene of interest are made. The primers listed in Table 8 are used to amplify the gene of interest from genomic DNA, whilst introducing restriction enzyme cutting sites at the 5' and 3' ends. Digestion of the PCR product and digestion of the vector with the restriction enzymes NcoI and EcoRI in buffer H for one hour at 37°C allows the creation of overlapping sticky ends. A DNA ligase is then used to allow the ligation of the gene of interest into the vector. After one hour at room temperature, ligation products are stored at 4°C until further use. Electro-competent cells of the respective deletion strains are prepared and transformed according to the protocol in 2.4. For the selection of successful transformants, LB agar plates containing Ampicillin are used.

2.9 Growth sensitivity analyses

2.9.1 Aerobic growth in the presence of potassium tellurite

For inoculation of 50 mL of LB broth, ~0.6 x 10^9 cells of overnight cultures are used. For the calculation, the optical density of a 1:10 dilution is measured at 600nm and the number of cells calculated (OD_{600nm} = 1 ≈ 1.2 x 10^9 cfu x mL^{-1}). The cultures are incubated at 37°C with shaking at 200 rpm. Every hour, the optical density is measured at a wavelength of λ = 600nm by using 1 mL of bacterial cultures until the OD reaches ~1.2. From then on, samples are diluted in LB at a 1:10 ratio. As a reference throughout the experiment, LB broth is used. Tellurite sensitivity is measured using the addition of 0.125 µg x mL^{-1} of K_2TeO_3 to 50 mL of LB. Where indicated, 100 µM DTT is added to cultures to eliminate oxidative stress caused by tellurite and its reduction. For complementation strains, L-arabinose (0.002% or 0.0002%) is added to the cultures to allow the expression of the gene from the pBAD plasmid.

Table 8: Primers used for the generation of cloning products

Gene:	Forward primer (5' - 3'):	Reverse primer (3' - 5'):
cstA	AAAACCATGGGTAATAAATCAGGGAAATACCT	AAAAAAGCTTTAGTGCGCGCCTTTCGCCTGCG
cydB	AAAACCATGGGTATCGATTATGAAGTACTACG	AAAAAAGCTTTAGTACAGAGAGTGGGTGTTGC
hcr	AAAACCATGGGTATGACGATGCCAACCTCACA	AAAAAAGCTTTATGCGAGAACAAGATCGCCCT
hmpA	AAAACCATGGGTCTTGACGCACAAACCATCGC	AAAAAAGCTTTACAGCACTTTATGCGGGCCGA
STM1808	AAAACCATGGGTTCCCACTTACGCATCCCGGC	AAAAAAGCTTCAGGCTTTTTTCTGTTGATAC
tehB	AAAACCATGGGTACCGTTCGTGACGAAAATTAC	AAAAAAGCTTCAGGCCGTTTTTCTCGCCAGCA
yeaR	AAAACCATGGGTGGCAAATTCCACAAAATCAT	AAAAAAGCTTTATTCTCTCCCGGCATGAATGA
ygbA	AAAACCATGGGTCCTGGTAAACGTATCGCTCG	AAAAAAGCTTCACTTCTTGCGCTGATATTTTT
ytfE	AAAACCATGGGTGCTTATCGCGATCAACCTTT	AAAAAAGCTTTATTCCCCGGCCAGCGCGCGTG
STM1273	AAAACCATGGGTAAACTCGATACCCGATTAAC	AAAAAAGCTTCATTTTGCGTGATACGGGGTCA

2.9.2 Preparation of aqueous NO solution

Oxygen-free nitrogen is used to sparge one litre of 1 M NaOH solution for a minimum of one hour. The solution is then scrubbed with NO gas (Sigma-Aldrich). A volume of 3 mL of distilled water with a pH of 3 are then sparged with 20 mL of NO gas. This will achieve a saturated NO solution with a concentration of 2 mM. Stored on ice, the solution is ready to use and stable for a maximum of 24 h (Mills et al, 2008).

2.9.3 Anaerobic sensitivity towards NO in minimal medium

Overnight cultures are grown in medium-sized glass vials filled to the top with 17 mL of MGM. The lids are sealed with parafilm to prevent oxygen from entering the vials. The cultures are grown statically overnight at 37 °C. A Hamilton syringe is used to inoculate 10 mL of MGM in Hungate tubes at a concentration of 5% (v/v). After inversion of the tubes, oxygen-free nitrogen gas is used to sparge the tubes for 5 min to remove residual oxygen in the head space. Incubation of the tubes occurs statically and the growth of the cultures is monitored by measuring the absorption at a wavelength of 590 nm at 1.5 hours

and every 30 min from then on. Once a value of 0.1 is reached, 40 µM aqueous NO is added and tubes are inverted briefly. L-arabinose at a concentration of 0.0002% is added to MGM for the growth of complementation strains.

2.10 β-galactosidase assay

Similar to the generation of constructs for the complementation study (see 2.8), the *STM1808* promoter region is PCR-amplified from genomic DNA, digested with restriction enzymes PstI and EcoRI and ligated into pMP220 (Zaat et al, 1987). This then allows monitoring the control of regulators such as NsrR on the binding to the promoter site and the expression of β-galactosidase as a consequence. For the PCR, the following forward *STM1808* prom F (AAAAGAATTCATACACACGCTCCTTCGGGA) and the reverse primer *STM1808* prom R (AAAACTGCAGAAGACATCTCCCTGATGTGG) are used.

2.10.1 Assay reagents

The following reagents are freshly prepared on the day of the experiment:

- Z buffer:
  0.06 M Na_2HPO_4 x 7 H_2O, 0.04 M NaH_2PO_4 x H_2O, 0.01 M KCl, 0.001 M $MgSO_4$, 0.05 M β-mercaptoethanol, pH adjusted to 7.0

- 0.1 M phosphate buffer:
  0.06 M Na_2HPO_4 x 7 H_2O, 0.04M NaH_2PO_4 x H_2O, pH adjusted to 7.0

- *o*-nitrophenyl-β-D-galactopyranoside (ONPG):
  4 mg x mL^{-1} in 0.1 M phosphate buffer

2.10.2 Experimental procedure

Fresh overnight cultures are used to inoculate 10 mL LB at a ratio of 1:100 and grown to mid-log (OD_{600nm} ~ 0.5). Cell growth is arrested by keeping the cells on ice for 20 minutes. Then 2 mL of culture are centrifuged for 10 minutes at 6,000 rpm at 4°C. The supernatant is discarded and cells are resuspended in 2 mL ice-cold Z buffer. The OD_{600nm} is measured and Z buffer is used as a reference.

The cells are diluted further by adding 0.5 mL cells to 0.5 mL Z buffer. To permeabilize the cells, 100 µL of chloroform and 50 µL 0.1% SDS are added and the mixture is vortexed. Equilibration of the mixture is achieved by placing it in a water bath at 28°C for 5 minutes. By adding 0.2 mL ONPG substrate (final concentration of 0.8 mg x mL^{-1}) to the mixture, the reaction is started. Once an adequate yellow colouring similar to LB broth is achieved (A_{420nm} ~ 0.6 - 0.9), 0.5 mL 1M Na_2CO_3 is added, vortexed and the time of addition noted down. The addition of Na_2CO_3 acts as a stop solution, raising the pH of the solution to 11 and stopping the enzymatic reaction. Of the reaction mixture, 1 mL is transferred to a centrifuge tube and centrifuged at 14,600 rpm for 5 minutes to remove cell debris. The supernatant is transferred to a cuvette and the A_{420nm} and A_{550nm} are measured. The absorbance at 420nm corresponds to the absorbance of *o*-nitrophenol, the product of the cleavage of ONPG performed by the β-galactosidase present. To eliminate false absorbance caused scattering by cell debris, the absorbance at 550nm is measured. At this wavelength, only cell debris scatters the light and the corresponding value is subtracted from the value obtained at 420nm. Miller units of enzyme activity are calculated using the following formula:

$$\text{Miller Units} = 1000 \times [(OD_{420} - 1.75 \times OD_{550})] / (T \times V \times OD_{600}).$$

The wild-type strain SL1344 is β-galactosidase-negative and therefore the wild-type strain containing the empty vector is used as a negative control for the experiment.

2.11 RNA extraction and quantification

SL1344 cultures are grown in 50 mL LB broth in conditions used for growth curves. After 3 hours of growth, 0.125 µg x mL^{-1} K_2TeO_3 is added to one set of the cultures and cultures are put back into the incubator. After 15 minutes, cells are harvested to achieve 4 OD units. Cell growth is arrested by the addition of a fifth volume of 5% (v/v) phenol (pH 4.3), 95% (v/v) ethanol and cell culture medium is removed by centrifugation at 3220 x g at 4°C for 10 min. Cell pellets are stored at -80°C until further use. RNA extraction from the cell pellets followed the protocol of the Promega SV40 Total RNA Isolation System kit. The

total RNA is treated with DNase (Ambion) to destroy the remaining DNA. DNA contamination is assessed using PCR or using the Experion RNA HighSens and RNA StdSens analysis kit (Bio-Rad). RNA purity and quantity are assessed using a NanoDrop (ThermoScientific) by comparing the ratio of OD_{260nm} to OD_{280nm}.

2.12 Real-time reverse transcription (RT-) PCR

After DNase treatment (see 2.10.1) 2 µg of total RNA are reverse-transcribed using SuperscriptII™ reverse transcriptase (Invitrogen) and random hexamers (Invitrogen) according to the manufacturer's instructions. For the real-time quantification of expression levels, gene-specific primers with approximate T_m of 60°C are designed (see Table 9 below). The average product size is chosen to be ~ 100 bp. Using the Bio-Rad CFX96™ Real-Time System, the quantification of a 5-fold dilution of the obtained cDNA is performed with the aid of the SensiMix™SYBR No-ROX kit (Bioline). The steps of the reaction can be found in Table 10. Three biological replicate with three technical replicates are analysed for each sample. For standardisation, dilutions of SL1344 chromosomal DNA obtained using a Qiagen Genomic DNA isolation kit are used. The threshold cycle values (C_t) for each gene are then normalised to the C_t of the *ampD* gene (Appia-Ayme et al, 2012).

2.13 Intracellular survival in macrophages

Cultures of RAW264.7 murine macrophages are maintained in DMEM (10% foetal bovine serum (FBS), 2 mM L-glutamine, 1x penicillin/streptomycin, 1x fungizone added) at conditions mimicking the human body (37°C, 5% CO_2). Macrophages are harvested by seeding 2 x 10^5 cells per well into a 24-well plate and incubating overnight. For the activation of the macrophages, 100 U IFN-γ are added 20-22 hours prior to the addition of bacterial cells. The bacteria are diluted in DMEM (without serum and antibiotics) to get a multiplicity of infection (MOI) of 10 (2 x 10^6 cfu x mL^{-1}). The macrophages are washed with PBS and 0.5 mL of bacteria is added, followed by a centrifugation step at 500 rpm for 2 min.

Table 9: Primers used for real-time PCR quantification

Gene:	Forward primer (5' - 3'):	Reverse primer (3' - 5'):
***ampD* qPCR**	ATGACGAAAAACCGTCCTTG	GGATCTATCGTTCCGGTGAA
***hmpA* qPCR**	TTAATGCTATCGCGGCCTAC	AATCTGGAAGCTGGTGTGCT
***nirB* qPCR**	CTACGGCTGCGAAGTGTGTA	AGTATCCTGTAGCGGCGTGT
***norV* qPCR**	GTCAGCACTACTGCGACGAG	CTGAACGGCGTCAGGATATT
***STM1808* qPCR**	GCTCTACCCCCTTTTTCACC	TTCCATCACGCAGAGTTGTC
***tehB* qPCR**	ACGCTGGATTTAGGATGTGG	CCATACTGGCCGGATTTTTA
***yeaR* qPCR**	ATACACGTTCCACGCCATTC	CCGAGAGACGAGGATAAACG

Table 10: Details of the PCR programs used

Use of the program:	**qPCR:**	
Description	temp [°C]:	t [sec]:
1) Initial denaturation	95	10 min
2) Denaturation	95	15
3) Annealing	58	15
4) Elongation	72	10
5) Repeat of steps 2) to 4)	39x	

The cells are placed back in the incubator for 30 min before the medium is replaced by a killing medium containing 100 µg x mL^{-1} Gentamicin. Intracellular bacteria are not affected and are protected inside the macrophages. After 90 min, the killing medium is replaced by a maintenance medium (10 µg x mL^{-1} Gentamicin). The time points for the harvest of intracellular bacteria are two and ten hours after the addition of bacteria. For harvesting, cells are washed with 1 x PBS after aspiration of the medium. 0.01% SDS in 1x PBS is then added, the plates are placed on a gel rocker for 20 minutes prior to pipetting up and down for 10 times to cause the lysis of the macrophages. The bacteria are diluted and plated onto LB agar plates. The number of colony-forming units (cfu) is assessed after an overnight incubation at 37°C and compared to the cfu at time points t_1 = 2 h and t_2 = 10h.

2.14 Continuous cultures

For continuous culture of bacterial strains the New Brunswick BioFlo® 310 reactor is run with a working volume of 1.2 L. Cells are grown aerobically in 10 mL LB overnight, before 50mL of MGN are inoculated (1% v/v). After aerobic overnight growth, the reactor containing MGN is inoculated and a further aerobic overnight incubation followed. Glycerol in the medium serves as the sole carbon source and electron donor. The terminal electron acceptor is nitrate. 1M NaOH and 0.1 M H_2SO_4 are used to ensure that the pH remains at 7.5 throughout the growth. After overnight aerobic growth, the air supply is limited from 100% to 0% and anaerobic state of the medium is achieved after one hour as measured with a dissolved oxygen probe. A feed containing MGN is set up and the dilution rate is set to 0.04 h^{-1}. To monitor cell growth and concentrations of nitrogen compounds, culture and gas samples are taken at regular intervals throughout the experiment (Rowley et al, 2012).

2.15 High-performance liquid chromatography & Gas Chromatography

To determine the nitrite and nitrate concentrations of continuous culture samples, high-performance liquid chromatography (HPLC) with an anion-exchange column Ion Pac AS22, 2 mm x 250 mm (Dionex, ICS-900) is used according to manufacturer's instructions (Rowley et al, 2012). The gas samples are analysed using a PerkinElmer Clarus® 500 Gas Chromatograph (GC) and Elite-PLOT Q (DVB Plot Column, 30 m length; internal diameter of 0.53 mm, oxygen-free nitrogen as carrier gas; 95% argon / 5% methane as make-up gas; temperatures according to manufacturer's instructions). A standard curve using N_2O gas standards (0.4, 100, 1000 ppm, StGas) is prepared. In order to calculate the total concentration of N_2O, an equal concentration of the gas is assumed for the head space and the culture solution. Henry's Law constant at 37°C of 0.453 is applied.

3 Analysis of the *Salmonella* Typhimurium transcriptome under nitrosative stress

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3.1 Introduction

Transcriptomics has been used as a powerful tool to understand how enteric bacteria such as *Salmonella* and *E. coli* respond to nitrosative stress and how this correlates with gene expression during infection. This chapter will review the NO relevant transcriptomic datasets in the literature and using datasets previously produced by the Rowley laboratory, to determine the core NO responsive regulon i.e. genes up-regulated during both endogenous and exogenous NO detoxification, for experimental analyses in future chapters.

3.1.1 *In vitro* analyses

The global transcriptional response of *E. coli* to the presence of *S*-nitrosoglutathione (GSNO) and to $NaNO_2$ during aerobic growth has first been investigated in 2004 (Mukhopadhyay et al, 2004). During exponential growth in LB-rich medium, either 0.1 mM GSNO or 1 mM $NaNO_2$ are added to the cultures and RNA extraction is performed five minutes post addition. Similar numbers of genes are up- and down-regulated for both compounds: 34 genes with more than a five-fold increase in expression levels in the presence of 1 mM GSNO are also at least fivefold up-regulated in the presence of 1 mM $NaNO_2$, including *hmpA*, *norVW* and *ytfE*. A list of genes that are significantly up-regulated in three or more studies can be found in Table 11. The expression of *hmpA*, *norV* and *norW* is increased by more than 30 fold under both conditions, stressing the importance of these systems for NO protection. A high number of genes encoding hypothetical proteins, e.g. *yfiD* and *ygbA*, show increased expression levels, indicating a yet to be characterised role during the response to nitrosative agents. Differences between expression levels of genes such as *nrfA* and *metL* between $NaNO_2$ and GSNO exposure highlight the differences in expression that are observed depending on the culture conditions and NO donor used. The group has examined the effect of different regulators in response to NO and hence determined the NO sensitivity level of deletion mutants of *fur*, *norR*, *oxyR* and *soxR*. All four regulators contain cysteines and some have bound iron that makes them susceptible to reactivity with RNS. Therefore, all four could play a role in initiating the gene expression changes observed in response to NO sources.

Table 11: Overview about genes significantly up-regulated in three or more studies. The experimental conditions for the studies were as follows:
Mukhopadhyay *et al*.: *E. coli*, LB medium, + O_2, overlap for genes up-regulated upon addition of 0.1 mM GSNO and addition of 1 mM $NaNO_2$.
Justino *et al*.: *E. coli*, minimal salts medium, - O_2, addition of 50 µM NO.
Flatley *et al*.: *E. coli*, minimal salts medium, overlap for genes up-regulated during aerobic and anaerobic growth after addition of 200 µM GSNO.
Filenko *et al*.: *E. coli*, minimal salts medium + 20 mM trimethylamine-*N*-oxide + 2.5 mM $NaNO_2$, - O_2, K-12 MG1655 wild-type compared to Δ*nsrR*.
Partridge *et al*.: ChIP-chip using a 3XFlag-tagged NsrR containing *E. coli* strain, - O_2.
Karlinsey *et al*.: *S*. Typhimurium, LB medium, + O_2, wild-type 14028 compared to Δ*nsrR*.
Eriksson *et al.*: *S*. Typhimurium, J774-A.1 murine macrophage infection, genes up-regulated at the eight hour time point are shown.
Roos & Klemm: bacteriuria-causing *E. coli*, urinary tract samples compared to in vitro-grown 83972 *E. coli* cells.
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Gene:	Annotation:	Mukhopadhyay *et al.*, 2004	Justino *et al.*, 2005	Flatley *et al.*, 2005 (+ O_2)	Flatley *et al.*, 2005 (- O_2)	Filenko *et al.*, 2007	Partridge *et al.*, 2009	Karlinsey *et al.*, 2012	Eriksson *et al.*, 2003	Roos & Klemm, 2006	Hautefort *et al.*, 2008
allD	ureidoglycolate dehydrogenase							x	x		x
citB	citrate utilization protein b							x	x	x	x
cspB	cold-shock protein							x	x		x
deoC	deoxyribose-phosphate aldolase	x							x		x
dgoK	2-oxo-3-deoxy-galactonate kinase						x		x		x
dgoR	galactonate operon transcriptional repressor						x		x		x
dsbA	protein disulfide isomerase I						x	x	x		
fhuF	ferric hydroximate transport ferric iron reductase	x						x			x
ftsJ	cell-division protein						x		x		x
hcp	hydroxylamine reductase			x	x	x	x	x	x	x	x
hcr	HCP oxidoreductase					x	x	x	x	x	x
hycA	formate hydrogenlyase regulatory protein						x	x			x
marB	hypothetical protein							x	x		x

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ndh	respiratory NADH dehydrogenase 2		x				x			x	x
norR/ ygaA	anaerobic NO reductase transcriptional regulator;						x	x	x		x
norV	anaerobic NO reductase flavorubredoxin	x	x	x	x		x		x		x
norW	NO reductase	x	x		x						
nrdH	glutaredoxin-like protein	x	x		x						x
nrdI	ribonucleotide reductase stimulatory protein	x	x								x
setB	proton efflux pump							x	x		x
soxS	DNA-binding transcriptional regulator	x	x						x		x
spvR	spv operon regulator							x	x		x
ssaE	secretion system effector							x	x		x
stfC	fimbrial outer membrane usher							x	x		x
STM0084	sulphatase							x	x		x
STM0769	hypothetical protein							x	x		x

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sufA	iron-sulphur cluster assembly scaffold protein	x	x				x				x
tehA	potassium-tellurite ethidium and proflavin transporter		x				x				x
ybeD	hypothetical protein		x						x		x
ycfR	outer membrane protein	x							x		x
ydcX	inner membrane protein						x		x	x	x
ydhC	inner membrane transport protein								x	x	x
ydiV	flagellar regulatory protein CgdR		x					x	x		x
yeaR	hypothetical protein		x			x		x		x	x
yehR	lipoprotein		x						x		x
yfhH	DNA-binding transcriptional regulator						x		x		x
ygbA	hypothetical protein	x				x	x	x		x	x
yhcN	outer membrane protein								x	x	x
yhcP	p-hydroxybenzoic acid efflux subunit AaeB								x	x	x

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yhcQ	p-hydroxybenzoic acid efflux subunit AaeA								x	x	x
yhcR	hypothetical protein								x	x	x
ylbE	hypothetical protein		x						x		x
yoaG	hypothetical protein							x		x	x
yohK	hypothetical protein						x		x		x
ytfE	iron-sulphur cluster repair di-iron protein	x	x			x	x	x		x	x

The role of the regulators OxyR and SoxRS is only minor as a deletion of either gene does not increase the sensitivity to either nitrosative compound. Like Fur, OxyR and SoxR react to the changes in iron level and to the presence of ROS. The authors conclude that the activation of Fur, OxyR and SoxRS is the collateral result of the reactivity of RNS with the iron or cysteines. Deletion of *fur* causes severe growth defects when cells are exposed to RNS, indicating that continuous de-repression of the Fur regulon results in different effects than temporary relief of repression during the exposure to RNS of a wild-type strain. The quick recovery of growth of a *norR* deletion strain is explained with the functional redundancy between NorV under NorR regulation and of HmpA, which is not part of the NorR regulon.

Changes in expression levels in response to different doses as well as over time are also investigated using primer extension assays and Northern blotting: The levels of *hmpA* and *norV* are highest at 1mM GSNO or $NaNO_2$, whereas the levels of the hypothetical protein-encoding *ygbA* stay the same irrespective of the RNS concentration. The induction of both *hmpA* and *ygbA* is noted at 0.1 mM GSNO or 0.1 mM $NaNO_2$, showing that both genes are highly responsive to RNS. In addition, their expression levels peak after 5 and at 90 minutes. These similarities further stress the possible involvement of YgbA during RNS defence.

Transcriptomic profile of E. coli during anaerobic growth and NO exposure

In 2005, the transcriptomic profile of *E. coli* grown anaerobically in the presence of 50 µM NO has been published (Justino et al, 2005). RNA extraction has been performed fifteen minutes after the addition of NO. Anaerobic conditions minimize the interference of oxygen with NO and an increased production of RNS as a result. A range of differences, but also similarities in comparison to the aerobic study have been found. The NO detoxification genes *hmpA* and *norVW* are again among the up-regulated genes. The Fur regulon shows increased levels, further stressing Fur's role as a NO-responsive regulator. In addition, FNR repression is relieved and FNR activated genes have significantly decreased expression levels. Therefore, FNR is added to the list of NO-sensitive regulators. The class of genes with the highest percentage of changes in expression levels belongs to genes encoding hypothetical proteins (17%), followed by genes involved in energy metabolism and transport and binding

(13% each). Transport and binding of metals as well as multi-drug transporters include *ydhK*, encoding a hypothetical fusaric acid resistance protein; *ydh*, encoding a putative drug-exporter that confers antibiotic resistance and *tehAB*, encoding for tellurite resistance. DNA repair is also of importance as the *nrd* operon is highly up-regulated. NrdEF encode for a class Ib ribonucleotide reductase facilitating the synthesis of deoxyribonucleotides for the synthesis of DNA.

Changes in genes encoding fimbrial-like proteins such as *fimG* are observed and a decrease in the flagellar biosynthesis gene *flgB* indicates that *E. coli* might reduce surface protein expression in response to NO. The interference of NO with [Fe-S] clusters results in a significant increase in genes of the *suf* operon and *iscA* and *iscR*. These genes allow for the new formation and repair of [Fe-S] clusters. The group has identified the importance of YtfE for dealing with NO stress and it has later been attributed to the repair of NO-induced damaged to [Fe-S] clusters (Justino et al, 2006; Justino et al, 2007).

In conclusion, this study has outlined that *E. coli*'s survival to NO exposure is due to a change in expression levels of NO detoxification enzymes (*hmpA*, *norVW*), increased expression of the assembly system of [Fe-S] clusters (*iscA*, *iscR*, *suf*), DNA repair (*nrd*), an increase in the energy production (*aceEF*, *lpdA*) all under the regulation of stress-responsive regulators Fur, FNR and SoxR. Only 10% of genes identified in this study are overlapping with the genes identified in the previous, aerobic study. The differences are attributed to the change of growth medium (minimal salts instead of LB-rich medium) and the difference in oxygen levels during growth (anaerobic, not aerobic growth).

Shortly after, the transcriptional adaptation of *E. coli* to the addition of 200 µM GSNO during aerobic and anaerobic steady-state growth in chemostats has been published (Flatley et al, 2005). As the bacterial growth in a chemostat is tightly controlled and less affected by parameters associated with batch culture, fewer genes show a significant change in expression levels after GSNO addition. A transient stop in oxygen uptake is observed and cell viability decreases by 70%, but adaptation to NO is achieved after five minutes. During aerobic growth, 17 genes are significantly up-regulated and only four show lower expression levels (*codB* (cytosine permease), *nac* (transcriptional

regulator), *ydcC* (conserved protein), *yeaD* (conserved protein)). The NO detoxification genes *hmpA* and *norV*, again, are among the group of up-regulated genes. In addition, six genes of the *met* operon are induced. The *met* genes are involved in the regulation and biosynthesis of methionine. A connection between methionine and NO is proposed to be the result of nitrosation of homocysteine (Hcy) by GSNO, which is then depleted from the synthesis pathway. Up-regulation of the *met* genes allows for the compensation of loss of Hcy. Deletion of individual *met* genes results in higher sensitivity towards GSNO during disc diffusion assays and providing exogenous methionine significantly increases the survival of wild-type cultures. The levels of *hcp*, encoding the hybrid cluster protein, and *yhaO*, gene for a putative transport protein, are also significantly increased.

During anaerobic growth, ten genes have significantly increased expression levels, when the expression levels of three genes are significantly reduced. Among the up-regulated genes, there is an overlap of six genes with the set of genes from the aerobic study: *norV*, *hcp*, *yhaO* as well as three met operon genes. In addition, *norW* and the gene for the ribonucleotide reductase accessory protein *nrdH* are also up-regulated. Both have already been highlighted in the Justino *et al*. study. In contrast to the previous two studies, no effect of Fur regulation is observed and it has been proposed that this is the result of the sufficient concentration of Fe in the defined medium. The influence of NorR regulation on *norVW* is further supported. Without the transcription of *hmpA* during anaerobic growth, it has been shown that expression of *norVW* is sufficient for NO detoxification. Furthermore, the genes of the methionine biosynthesis provide additional protection against NO. Although regulation of *hcp* via FNR and NarLP has previously been identified, the role of Hcp is still unclear (Van Den Berg et al, 2000).

A close link between NsrR regulated genes and NO defence has been shown in the past through gene regulation of *hmpA*, *ytfE* and *ygbA*, encoding for a hypothetical protein (Bodenmiller & Spiro, 2006). A comparison of gene expression levels between an *E. coli* K-12 MG1655 parent and a *nsrR* deletion strain has been performed with the aim to evaluate the NsrR regulon (Filenko et al, 2007). Relief of NsrR-mediated repression of gene expression is observed

for several genes and operons during anaerobic growth, including *hcp-hcr* (hydroxylamine reductase and oxidoreductase), *yeaR-yoaG* (putative tellurite resistance), *nap* (nitrate reductase), *hmpA* (NO dioxygenase), *ygbA* (hypothetical protein), *nrfA* (cytochrome c nitrite reductase) and *ytfE* (iron-sulphur repair). Although the *nap* operon encoding for a nitrate reductase has been shown to be NsrR regulated, the other *E. coli* nitrate reductase genes *narGHIJ* and the nitrate reductase genes *nirBD* are not affected by NsrR. The tight regulation of *hcp-hcr* has further highlighted the potential of Hcp during NO detoxification.

A further analysis with the aim to identify direct NsrR binding sites across the *E. coli* genome has been performed using ChIP-chip (Partridge et al, 2009). Of 62 identified NsrR-binding sites, 33 have previously been identified, including *hcp-hcr*, *hmpA*, *nrfA*, *yeaR-yoaG*, *ygbA* and *ytfE*. In addition, several sites are located within promoter sequences of genes whose expression is significantly increased during the infection of the human urinary tract such as *tehAB* (Roos & Klemm, 2006). New NsrR targets are located in six categories of genes: Motility (e.g. *fliA*, *fliL*), carbon and energy metabolism (e.g. *aceF*, *ndh*), NO defence (*norR*, *norV*), proteolysis (e.g. *clpB*, *ptrA*), transport processes (e.g. *dsdX*, *yhfC*) and stress responses (*sodB*, *sufA*). The effect of NsrR on motility is further demonstrated as the absence of NsrR results in an increase in motility as did exposure to NO, a response which is not the mediated by HmpA. Additionally, surface attachment of an uropathogenic *E. coli* strain increases when *nsrR* is deleted or when the wild-type strain is exposed to NO. Therefore, NsrR binding represses motility and attachment until *E. coli* is exposed to NO, signalling arrival of the bacteria within the human body.

Further analysis of the NsrR regulon of *S.* Typhimurium has been published recently, and although it has not available to inform the experiments conducted in this thesis, the results have been included here for completion. This study has analysed the role of NsrR regulated genes for aerobic NO detoxification by *Salmonella* (Karlinsey et al, 2012). NsrR-regulation is defined using the comparison of gene expression levels across the whole genome of a wild-type strain 14028 with levels in an isogenic *nsrR* deletion strain. The results indicate that apart from *hmpA*, more than 30 operons are up-regulated. This list of up-

regulated operons include among others, *hcp-hcr*, *yeaR-yoaG*, *ygbA*, *ytfE* and *STM1808*. Bioinformatics have further revealed NsrR-binding sites upstream of *tehAB*, but confirmation by qRT-PCR is only possible for operons identified using microarray analysis. The deletion strains of single genes have been created and sensitivity towards the NO-releasing compound Spermine-NONOate has been tested in LB medium in the presence of oxygen. Confirmation of *hmpA* sensitivity is shown along with impaired growth of Δ*STM1808*. No sensitivity of the other deletion strains has been observed. In M9 minimal medium the sensitivity of strains increases. A contribution of HmpA, Hcp, YgbA and STM1808 is proposed to help with the aerobic resistance of *S*. Typhimurium against nitrosative stress.

3.1.2 *In vivo* analyses

In 2003, Eriksson *et al.* have published the first comprehensive analysis of the *S*. Typhimurium transcriptome during infection of murine macrophages. This study identifies more than 900 genes that are significantly up-regulated inside macrophages compared to a LB culture. Of relevance to this study they include *hmpA*, *norV* and its regulator *norR*. Apart from *hmpA*, three other NsrR-regulated genes also have high expression levels: *hcp*, *hcr* and *ytfE* are significantly up-regulated after four (*hcp*, *hcr*) and eight hours (*ytfE*) respectively, indicating the potential role of the encoded proteins in the response towards nitrosative and peroxynitrite stress in macrophages. As mentioned in the main introduction, the release of NO by macrophages occurs at approximately eight hours after the uptake into the intracellular vesicles, whereas release of ROS occurs after approximately four hours. Therefore, the changes in gene expression of *hcp* and *hcr* relate to the release of ROS and the changes in gene expression of *ytfE* are the result of the release of NO.

Through examining the expression profiles this group has established that the environment of the SCV is high in potassium, but low in magnesium and phosphate. No induction of iron acquisition or storage genes is observed, although it is known that NO mimics the depletion of iron by e. g. by interfering with Fe-Fur complexes (Crawford & Goldberg, 1998a). Due to the expression of operons for e. g. succinate dehydrogenase, it is concluded that oxygen is

available in the SCV. In correlation with previous reporter gene assays, the induction of various SPI-2, SPI-3 and SPI-5 genes (e.g. *ssrAB*, *mgtBC* and *pipABD*, respectively) has been shown.

In comparison to the intracellular macrophage environment, in the epithelial cell (HeLA) intracellular environment is free of NO as epithelial cells do not possess iNOS needed for NO production. In HeLa cells, the transcriptional levels of the known NO detoxification and repair genes *hmpA*, *norV*, *nrfA* and *ytfE* do not change significantly (Hautefort et al, 2008). Thus they play a specific role during exposure to oxidative and nitrosative stress in comparison to a more general role in *S*. Typhimurium infection.

The transcriptomic profile of the asymptomatic bacteriuria-causing *E. coli* strain 83972 has been performed to investigate which genes are expressed during colonization of the urinary tract (Roos & Klemm, 2006). The study reveales an increase in expression levels of genes from the *nar*, *fdn* and *met* operons in comparison with *in vitro*-grown 83972 cells. Iron acquisition is of high priority since the concentration of soluble iron is low in the urinary tract and almost all genes with a role in iron transport and uptake are up-regulated (*iucABCD*, *sitABCD*, *iroBCDEN*, *chu* operon). Up-regulation of the *nar* and *fdn* operons is reflecting the high concentration of nitrate in human urine and correlates to increased levels of *nirBC* and *nrfA*, which allow for reduction of nitrite. In accordance with reports about NO tolerance and virulence of uropathogenic *E. coli* strains, up-regulation of *hmpA*, *ytfE* and *norV* has been reported. This could be an explanation for persistent growth of this *E. coli* strain in the urine without eradication by the immune system. In addition, it allows for the use of nitrate for respiration without the accumulation of toxic endogenous NO.

To draw a comparison between *in vitro* and *in vivo* studies has proven difficult. Both systems have advantages, but are too different to compare directly. It is also difficult to compare *in vitro* experiments from different laboratories due to the impact even subtle changes in environment can have on transcription.

Nevertheless, the recent study on NsrR regulation highlights genes that are also reported to be up-regulated during urinary tract infections caused by *E. coli* as well as have been found to be up-regulated during intracellular survival of *Salmonella* (Eriksson et al, 2003; Roos & Klemm, 2006). Overlaps between *in*

vitro and *in vivo* studies, validate the importance of particular genes for NO detoxification. In summary, the literature search has revealed that in particular three genes are of importance for NO defence in enteric bacteria, namely *hmpA*, *norV* and *ytfE*. In addition, several other genes have been implicated in detoxification of NO: *hcp-hcr*, *ygbA*, *yeaR-yoaG*, *nrfA* and *STM1808*. Their specific roles during NO exposure and the infection process of *Salmonella* in particular, however, have not been fully investigated.

3.2 Aim

The aim of this study is to make a comparative analysis of the *Salmonella* Typhimurium transcriptome during endogenous and exogenous NO exposure and to determine a core NO detoxification regulon. These studies have been carried out previously by members of the Richardson and Rowley groups. One study highlights the transcriptional response to an endogenous source of NO produced as a side product of nitrate metabolism. The other experiment measures the transcriptional response to aqueous NO addition to the culture. The hypothesis is that genes up-regulated in both conditions would form a core set of genes or regulon that allow *Salmonella* to deal with all aspects of NO detoxification. This core NO regulon would be further investigated in other chapters.

3.3 Experimental design

For the identification of new mechanisms on how *Salmonella* detoxifies NO, several aspects need to be considered: When using microarray analysis for the identification, every experimental condition leads to the increase in gene expression of some genes that have no involvement in NO detoxification in particular, but might belong to a general stress response or to a metabolic pathway affected by nitrosative stress or are induced under specific growth conditions.

The procedure for the microarray experiment has been described previously and was performed by members of the Richardson and Rowley groups (Appia-Ayme et al, 2011). Continuous culturing and the analysis of nitrate, nitrite and

nitrous oxide concentration follow the protocol in 2.14 and 2.15. The preparation of aqueous NO solution has been described in 2.9.2. The extraction of RNA has been described in 2.11 but MGN medium was used for continuous growth and 2.12 describes the procedure for quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). The analysis presented here is based on the experimental data generated by others, mostly by Dr Paul Mills (NO) and Dr Gary Rowley (N_2O).

3.3.1 Endogenous NO production

The production of NO is not solely the result of exposure within macrophages, but it also occurs as a product of nitrate respiration as part of denitrification. If *S.* Typhimurium is provided with a limited source of glycerol, a high concentration of nitrate as an electron acceptor and is grown anaerobically, nitrate is predominantly converted into nitrite (Figure 10). Nitrite is further reduced to NO. This endogenous NO production is potentially toxic for the cells and its accumulation should be avoided. Therefore the NO produced is reduced to N_2O. Figure 6 (page 50) shows the denitrification pathway and the genes involved. N_2O readily diffuses out of cells, reducing its toxicity potential for bacteria like *E. coli* and *Salmonella* that lack a nitrous oxide reductase. Additionally, extracellular N_2O can be measured in the headspace of bioreactors and N_2O concentration of the gas and liquid phase can be calculated (Rowley et al, 2012).

For the analysis the gene expression levels at three different time points have been determined: At five hours of continuous growth, aerobic growth takes place as air is still supplied into the chemostat. No nitrate is used and hence no nitrite or N_2O production is seen (Figure 10). No induction of nitrate respiration genes is expected. The gene expression levels at this time point provide the basis for comparison with later time points.

At 24 hours, the air supply is turned off and a switch to anaerobic respiration using nitrate is observed (Rowley et al, 2012). After three to four changes of vessel volume (i.e. ~80 to 120 hours), a steady growth state is achieved. Further time points for RNA sampling are chosen at the beginning (80 h) and the end of the steady state of the experiment (120 h).

The comparison of genes differently induced after the switch from aerobic to anaerobic respiration should include genes involved in denitrification, in the metabolic switch from aerobic to anaerobic growth, but also those aiding in dealing with the toxicity and reactivity of NO.

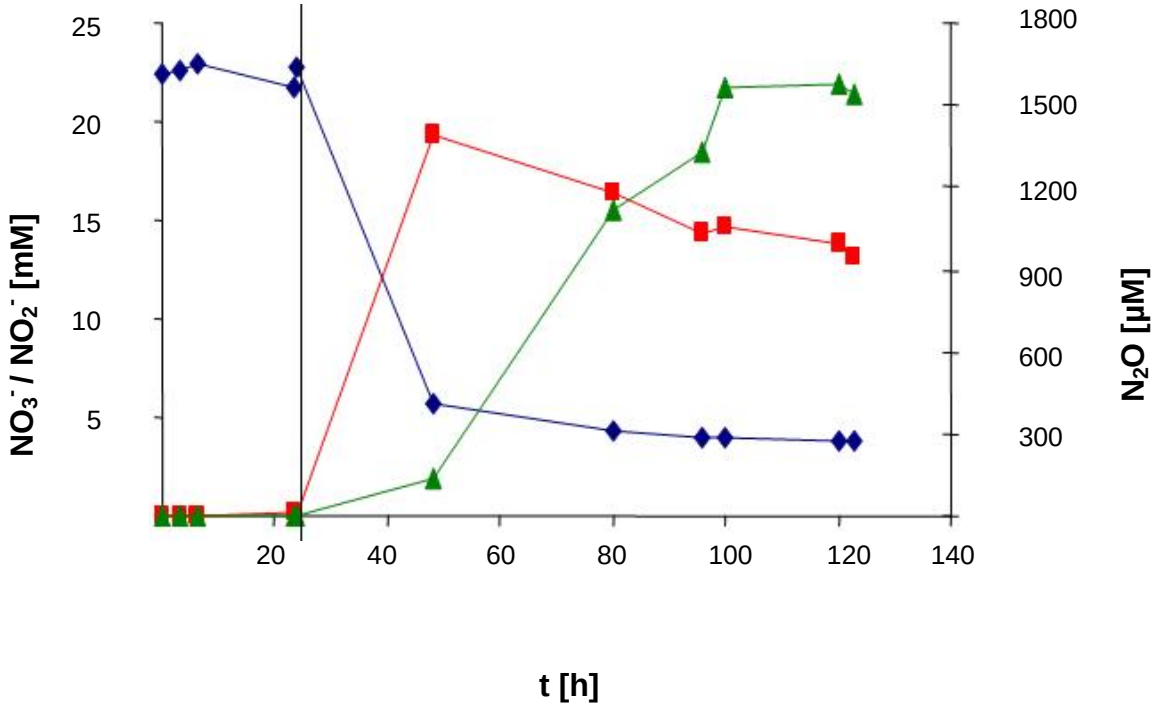


Figure 10: Nitrate, nitrate and nitrous oxide concentrations during continuous growth of *Salmonella* Typhimurium SL1344 under glycerol-limitation and nitrate-sufficiency (Rowley et al, 2012).

Nitrate concentrations (in mM) are shown in blue diamonds, nitrite [mM] in green triangles, respectively. Nitrous oxide concentrations range in µM levels and are shown in red squares. The black line indicates the switch from aerobic to anaerobic growth with oxygen being consumed within one hour.

3.3.2 Exogenous NO exposure

During infection, *Salmonella* encounters exogenously produced NO within the phagosomes of macrophages. Although it has been shown that *Salmonella* blocks the fusion of the lysosome with the phagosome (Uchiya et al, 1999), the presence of NO can still be detected within murine macrophages (Webb et al, 2001). Within non-activated macrophages, the nitrosative burst occurs eight hours after bacterial uptake (Eriksson et al, 2000). However, when macrophages are activated with IFN-γ before bacterial exposure, the burst occurs after four hours (Webb et al, 2001). The reported NO concentrations within macrophages range from 20 µM (Eriksson et al, 2000) to 40 µM, rising to 90 µM for activated macrophages (Vazquez-Torres et al, 2000a).

Here, *Salmonella* is grown anaerobically in a minimal salts medium and the addition of 40 µM aqueous NO solution is used to mimic conditions of NO release in the macrophages.

3.4 Results

3.4.1 Expression profile during endogenous NO production

In order to cause endogenous NO production by Salmonella, the bacteria are continuously grown anaerobically in the presence of nitrate as an electron acceptor. As a control time-point for expression, RNA samples are collected at 5 hours, where growth in MGN occurs aerobically. This condition ensures the absence of nitrate respiration and no N_2O, an indicator for NO production, is detected. Steady state of the cultures is reached after 80 to 120 hours, when the cultures have undergone a switch from aerobic to anaerobic growth, using nitrate and increasing nitrite and N_2O concentrations. At these points *Salmonella* are undergoing nitrate respiration and are therefore dealing with nitrosative stress as a consequence of NO and N_2O in the medium.

For data analysis, the cut-off criteria are set to only include genes whose expression is ≥ five fold up-regulated at both 80 and 120 hours in comparison to 5 hours, with an FDR of 0.05. After this filtering 45 genes are significantly up-regulated (Table 16).

Table 12: *Salmonella* Typhimurium genes up-regulated during NO (N_2O) generating conditions
Genes presented are a minimum of five fold up-regulated ($p<0.05$) during steady state in nitrate-sufficient (22 mM), glycerol-limited (5 mM) conditions. Significance is derived from a minimum of three biological replicates. Gene annotations from the KEGG database are shown.

gene:	annotation:	fold change for each operon member: 5 h to 80 h	5 h to 120 h
agp	glucose-1-phosphatase/inositol phosphatase	7	5
aphA	acid phosphatase/phosphotransferase	6	7
cstA	carbon starvation protein	7	6
cydAB	cytochrome d terminal oxidase polypeptide subunit I & II	14; 13	10; 10
ego	ABC-type aldose transport system ATPase component	5	8
fdnGHI	formate dehydrogenase-N subunits	17; 13; 8	21; 17; 9
flgFI	flagellar basal body rod protein; flagellar basal body P-ring protein	15; 9	7; 6
glpKX	glycerol kinase; ructose 1,6-bisphosphatase II	18; 5	17; 6
hcr	HCP oxidoreductase	77	67
hmpA	nitric oxide dioxygenase	172	121
hypB	hydrogenase nickel incorporation protein HypB	8	8
metABF	homoserine O-succinyltransferase; cystathionine γ-synthase; 5,10-methylenetetrahydrofolate reductase	10; 14; 12	6; 8; 7
narGHIJK	nitrate reductase 1 subunits	203; 54; 26; 80; 73	179; 50; 25; 82; 57
norV	anaerobic nitric oxide reductase flavorubredoxin	8	4
ompF	outer membrane protein F precursor	23	14
purF	amidophosphoribosyltransferase	5	5
putA	bifunctional PutA protein; bifunctional in plasma membrane proline dehydrogenase and pyrroline-5-carboxylate dehydrogenase OR in cytoplasm a transcriptional repressor; contains frameshifts (1) colibase information	19	10
sbmC	DNA gyrase inhibitor	7	10
sfbABC	ABC transporter ATPase/permease	14; 8; 8	14; 11; 8

gene:	annotation:	fold change: 5 h to 80 h	5 h to 120 h
stcA	fimbrial-like protein	6	9
thiFH	thiamine biosynthesis proteins	6; 9	5; 7
udg	UDP-glucose/GDP-mannose dehydrogenase	6	8
ybdHL	hypothetical protein (uncharacterised oxidoreductase in EC); aminotransferase	14; 9	11; 7
ydeV	autoinducer-2 (AI-2) kinase	5	6
ydeZ	sugar transport protein	5	7
yeaR-yoaG	hypothetical proteins	30; 10	41; 12
yfbG	bifunctional UDP-glucuronic acid decarboxylase/UDP-4-amino-4-deoxy-L-arabinose formyltransferase	7	9
ygbA	Hypothetical protein	11	12
yneA	sugar transport protein	7	8
ytfE	iron-sulphur cluster repair di-iron protein	43	36

Using qRT-PCR, it is possible to also include the changes in gene expression of *norV* since this gene has not been included on the probes of the microarray slide. The changes in gene expression for *norV* levels can be seen in Figure 11. The endogenous NO induced list includes a range of NsrR- and FNR-regulated genes such as *hmpA*, *hcr*, *yeaR-yoaG*, *narGHIJK*, *cydAB* and *ygbA*. The growth conditions based on high nitrate and low glycerol concentrations are reflected in the significant expression levels changes of genes known to be involved or putatively involved in glycerol metabolism (*glpFKQX*, *ybdH*) and in line with previous reports, the expression levels of *fdnGHI* are increased in response to nitrate (Overton et al, 2006a).
The known and proposed NO detoxification genes *hmpA*, *norV* and *ytfE* are highly expressed and all three genes have previously been highly up-regulated in response to nitrosative stress (Filenko et al, 2007; Flatley et al, 2005; Justino et al, 2005; Karlinsey et al, 2012; Mukhopadhyay et al, 2004).
The high expression levels of above 120 for *hmpA* reflect the need to activate NO detoxification and repair mechanisms to avoid accidental poisoning by NO. Although *in vitro* experiments have shown low activity levels of *hmpA* anaerobically, deletion of *hmpA* results in reduced intracellular survival of *S*. Typhimurium in human macrophages (Mills et al, 2008; Stevanin et al, 2002). Therefore, the importance of *hmpA* during anaerobic NO detoxification should not be underestimated.

The levels of *narG* also peak at high values of above 150. This highlights the importance of NarG as a nitrate reductase for the growth and survival of *Salmonella* in glycerol-limited and nitrate-sufficient medium.

In comparison with previous studies, several genes identified here overlap with results of other groups: Amongst up-regulated genes after the addition of GSNO as a nitrosylating agent during continuous anaerobic culturing of *E. coli* are several methionine biosynthesis genes (*metABF*) (Flatley et al, 2005). A link between the *met* genes and NO detoxification has been identified to lie in the nitrosylation of homocysteine, also linked to *hmpA* expression as previously described (De Groote et al, 1996; Membrillo-Hernández et al, 1998).

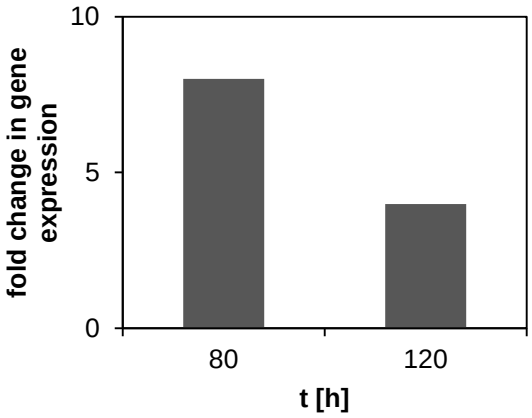


Figure 11: Gene expression levels of *norV* at 80 h and 120 h of anaerobic growth in MGN
Gene expression levels have been normalised to levels of *ampD* and to values at 5 h where cells have been growing under aerobic conditions.

The high levels of expression of cytochrome d oxidase genes *cydAB* have previously been reported during anaerobic growth of *E. coli* after the addition of trimethylamine-*N*-oxide (Filenko et al, 2007) and in *S. aureus* (Richardson et al, 2006). The increased expression of this alternative *bd*-type ubiquinol oxidase, usually functioning during aerobic growth, might allow survival during the increased stress of NO onto the respiratory chain whilst compensating for the decreased functioning of other oxidases (Stevanin et al, 2000).
In general, the comparison between studies using different NO donors has been difficult, especially if different media and growth conditions have been used. However, there is some overlap with regards to genes such as *hcr*, *hmpA* (NO dioxygenase), *norV* (NO reductase) and *ytfE* (iron-sulphur repair) across several studies, indicating the importance and potential role of their proteins for NO detoxification.

3.4.2 Expression profile during exposure to 40 µM NO

The addition of 40 µM NO mimics the NO concentration generated by iNOS in human macrophages during the nitrosative burst. During the experiment, *Salmonella* wild-type cells are exposed to 40 µM NO in anaerobic conditions and samples for RNA analysis are taken before as well as ten minutes after the addition of NO. For further analysis, a FDR cut-off of 0.05 has been chosen for gene expression levels at 10 minutes in comparison to 0 minutes. After 10 mi-

nutes of NO exposure, the expression levels of 139 genes are significantly increased (Table 13).

Of the three previously identified NO detoxification genes, *hmpA* and *norVW* (*STM2840*, *ygbD*) are up-regulated. The proposed [Fe-S] cluster repair gene *ytfE* is also among the up-regulated genes, supporting its potential role of NO-caused damage repair.

The regulation by NsrR and FNR marks one of the largest groups of up-regulated genes, which include NO detoxification (*hmpA*, *ytfE*), pyruvate dehydrogenases (*aceEF*, *lpdA*, *pdhR*) and putative tellurite resistance genes (*tehAB*, *STM1808*, *yeaR*).

The NsrR-regulon comprises the repression of the *ytfE* gene (Filenko et al, 2007) and also regulates *tehB* in *Salmonella* spp., a homolog encoding for a tellurite resistance protein that is also found in the genome of *E. coli* (Rodionov et al, 2005). [Fe-S] clusters are prone to damage by NO which results in de-repression of NsrR regulated genes in *E. coli* after endogenous NO exposure (Pullan et al, 2007). Other regulators playing a role in the nitrosative stress response and coordinating the change of gene expression are FNR and Fur that also contribute to gene expression changes in this study (Constantinidou et al, 2006; Overton et al, 2006a; Pullan et al, 2007).

The addition of nitrate or nitrite respectively causes the up-regulation of the several operons including *hcp-hcr* and *nirBDC* that belong to the FNR-activated genes. The *hmp* and the *ytfE* operon are among the operons that are repressed by FNR, but the addition of either nitrite or nitrate causes an activation of the gene expression. This indicates the putative regulatory mechanism, which ensures that the expression of *hmp* is not disabled during the exposure to RNS. The exposure to NO negatively affects the [Fe-S] clusters of FNR and renders its repression ineffective, resulting in the expression of the protective flavohaemoglobin HmpA. Fur is also affected by the presence of nitric oxide, potentially by reactivity of NO with protein-bound iron (Pullan et al, 2007).

Table 13: *Salmonella* Typhimurium genes up-regulated during exogenous NO addition
Genes presented are a minimum of two-fold up-regulated ($p<0.05$) 10 min post NO addition to anaerobic cultures when compared to 0 min. Data shown is the mean of five biological replicates. Annotations have been supplied from the KEGG or the NCBI database where indicated by an asterisk.

gene:	**annotation:**	**fold change:**
aceEF	pyruvate dehydrogenase subunit E1; dihydrolipoamide acetyltransferase	30; 18
avrA	secreted effector protein	12
bfd	bacterioferritin-associated ferredoxin	8
clpB	protein disaggregation chaperone	9
cstA	carbon starvation protein	4
cueO	multicopper oxidase	32
cydB	cytochrome *d* terminal oxidase polypeptide subunit	4
cyoDE	cytochrome *o* ubiquinol oxidase subunit IV; protohaem IX farnesyltransferase	15; 9
entABCE	2,3-dihydroxybenzoate-2,3-dehydrogenase; 2,3-dihydro-2,3-dihydroxybenzoate synthetase; isochorismate synthase; enterobactin synthase subunit E	26; 63; 21; 26
fbaB	fructose-bisphosphate aldolase	5
fdoI	formate dehydrogenase-O subunit γ	2
fepABC	outer membrane receptor; iron-enterobactin transporter periplasmic binding protein; iron-enterobactin transporter ATP-binding protein	19; 4; 5
fes	enterobactin/ferric enterobactin esterase	14
fhuACE	ferrichrome outer membrane transporter; iron-hydroxamate transporter ATP-binding subunit; ferric-rhodotorulic acid outer membrane transporter	18; 8; 6
fliC	flagellin	3
fljB	flagellin	2
foxA	ferrioxamine receptor	4
fxsA	cytoplasmic membrane protein*	2
gcd	glucose dehydrogenase	8
gpmA	phosphoglyceromutase	24
hcr	HCP oxidoreductase	2

gene:	annotation:	fold change:
hin	DNA-invertase	3
hmpA	nitric oxide dioxygenase	185
hscB	co-chaperone	2
hslJ	heat-inducible protein	2
ibpAB	heat shock protein/chaperone	6; 10
ilvC	ketol-acid reductoisomerase	17
iroBCDEN	glycosyl transferase family protein; ABC transporter; enterochelin esterase-like protein; hydrolase; outer membrane receptor FepA	54; 24; 9; 9; 77
katE	hydroperoxidase II	6
lpdA	dihydrolipoamide dehydrogenase	9
msrA	methionine sulphoxide reductase A	5
napBG	citrate reductase cytochrome *c*-type subunit; quinol dehydrogenase periplasmic component	4; 2
ndh	respiratory NADH dehydrogenase 2	19
nifSU	cysteine desulphurase; scaffold protein	4; 6
norVW	anaerobic nitric oxide reductase flavorubredoxin; nitric oxide reductase	613; 204
nrdA	ribonucleotide-diphosphate reductase subunit α	4
osmY	hypothetical protein	14
pdhR	transcriptional regulator	8
phoH	riboflavin biosynthesis protein	10
poxB	pyruvate dehydrogenase	5
rmf	ribosome modulation factor	3
sitABCD	periplasmic-binding protein, ATP-binding protein; permease	88; 94; 80; 49
sodA	superoxide dismutase	61
STM0382	permease	2
STM0412	hypothetical protein	2
STM0497	hypothetical protein	5
STM0771	ABC-type cobalamin/Fe^{3+}-siderophore transport component	8
STM1250	hypothetical protein	81

gene:	annotation:	fold change:
STM1331	No information available	2
STM1586	hypothetical protein	24
STM1808	hypothetical protein	195
STM1868A	lytic enzyme	169
STM1873	hypothetical protein	2
STM2314	chemotaxis signal transduction protein	3
STM2405	indolepyruvate decarboxylase	3
STM2697	phage tail-like protein	3
STM2923	hypothetical protein	2
STM3160	inner membrane protein	5
STM3362	hypothetical protein	4
STM3698	permease	2
STM3766	hypothetical protein	2
STM4552	inner membrane protein	26
STM4562	hypothetical protein	6
sufCDS	cysteine desulfurase subunit ATPase; cysteine desulfurase activator complex subunit; bi-functional cysteine desulfurase/selenocysteine lyase	14; 13; 11
talA	transaldolase A	5
tcp	methyl-accepting transmembrane citrate/phenol chemoreceptor	5
tehAB	potassium-tellurite ethidium and proflavin transporter; tellurite resistance protein	4; 6
treAF	trehalases	4; 10
uspB	universal stress protein	5
ybaY	hypothetical protein	5
ybfA	hypothetical protein	5
ybgET	hypothetical proteins	3; 4
ybhRS	transporter proteins	3; 2
ybiH	DNA-binding transcriptional regulator	4
ybjP	lipoprotein	2

gene:	annotation:	fold change:
ycfR	outer membrane protein	18
ycgB	SpoVR family protein	7
ydcK	nucleoside-diphosphate-sugar pyrophosphorylase	3
ydiU	hypothetical protein	3
yeaG	serine protein kinase	18
yeaR-yoaG	hypothetical proteins	27; 7
yehYZ	ABC-type proline/glycine betaine transport system permease component; transporter	3; 5
yfaE	2Fe-2S ferredoxin	2
yfhFP	putative iron-sulphur cluster insertion protein ErpA*; DNA-binding transcriptional regulator IscR	3; 16
ygaM	hypothetical protein	8
ygbAI	hypothetical protein; regulatory protein	14; 6
yghU	glutathione *S*-transferase	6
ygiN	hypothetical protein	3
yhbO	intracellular proteinase	4
yhcN	outer membrane protein	5
yhgGHI	ferrous iron transport protein C; gluconate periplasmic binding protein; Fe/S biogenesis protein NfuA	4; 2; 3
yhhA	hypothetical protein	8
yiaG	transcriptional regulator	14
yifA	transcriptional regulator HdfR	2
yjcB	inner membrane protein	4
yohJ	hypothetical protein	4
ypeC	hypothetical protein	6
yqiCG	glutathionylspermidine synthase*	2; 6
ytfE	iron-sulphur cluster repair di-iron protein	137

The Fur regulon includes the *suf, sod* and *ent* operons as well as *cyoA*, *hmpA*, *norW* and *ytfE* (Hernández-Urzúa et al, 2007; Justino et al, 2006; Justino et al, 2005; Kumar & Shimizu, 2011; Patzer & Hantke, 1999). These genes are all up-regulated during exogenous NO exposure, stressing the important role of Fur regulation in nitrosative stress.

The presence of NO has been reported to cause disruptions in the bacterial respiratory chain. One method of compensation for this is expression of HmpA (Stevanin et al, 2000). An increased level of three cytochrome oxidase genes (*cydB*, *cyoDE*) has been noted in this study that could allow compensating for the vulnerability of cytochrome oxidases to NO damage.
NO also causes the nitrosylation of ribonucleotide reductase. As a consequence, the formation of deoxyribonucleotides is disrupted, leading to a lack of synthesis and repair of DNA (Lepoivre et al, 1991). The transcription levels of *nrdA*, a gene encoding for such a ribonucleotide reductase, are increased and similar findings have been made in several other microarray studies (Filenko et al, 2007; Flatley et al, 2005; Justino et al, 2005; Mukhopadhyay et al, 2004). Increased expression of *nrdA* could allow for sufficient repair of DNA during nitrosative stress conditions.

A large number of genes involved in metal homeostasis and [Fe-S] cluster formation are highly up-regulated. NO readily replaces iron in [Fe-S] clusters, leading to conformational changes of proteins and the release of free iron, which fuels the Fenton reaction, resulting in the release of hydroxyl radicals. Therefore, protein degradation is increased, but also the levels of ROS within the cell. Hence, bacteria need to ensure that iron is stored or bound using ferritins and siderophores. In addition, [Fe-S] cluster formation systems are activated to a higher level to allow the incorporation of iron into new [Fe-S] clusters as a way to sequestering free iron and to allow the formation of new proteins containing [Fe-S] clusters. With regards to iron storage, the levels of the Bfd, forming a complex with the bacterioferritin Bfr are increased. The release of Fur repression upon Fur negatively regulated gene clusters is eminent for operons involved in metal ion uptake (*sit*) as well as for siderophores (*iro*, *ent*). Similar relief of Fur repression has been reported

previously (Mukhopadhyay et al, 2004), underlining the important role of Fur in response to RNS. With regards to [Fe-S] clusters, the involvement of three pathways for their formation has been shown. Of the three pathways, members belonging to the nitrogen fixation operon (*nifSU*), the iron sulphur cluster (*iscR*) as well as from the sulphur formation operon (*sufCDS*) are highly expressed. According to previous reports in the literature, mainly the Suf pathway is implicated to be involved under stress conditions (Ayala-Castro et al, 2008), but according to the presented results, the other two pathways also play a role.

In conclusion, a wide range of genes related to metal ion homeostasis and [Fe-S] cluster formation are up-regulated to reduce potential damage caused by free iron, to continue [Fe-S] cluster formation as well as to maintain essential iron concentrations. These findings overlap with previous studies, where [Fe-S] cluster repair genes from the *suf* operon and *ytfE* have been highly up-regulated (Justino et al, 2005). Induction of *hmpA* and *norV* genes further ensures that NO detoxification takes place and increased transcription levels have been reported in previous *E. coli* studies (Filenko et al, 2007; Justino et al, 2005; Mukhopadhyay et al, 2004). Other genes up-regulated reflect the nitrosylation properties of NO and might allow counteracting these effects by increasing the number of functional proteins being produced or allowing for repair of damaged DNA.

3.5 The core NO regulon

To understand, which genes are absolutely required to detoxify NO from any source, we determine the overlap in genes which are up-regulated in both of these transcriptomic datasets. This set of genes hence is referred to as the core NO regulon.

The overlap between the two datasets reveals nine genes to be significantly up-regulated under both conditions and their respective expressional regulators are listed in Table 14. Their functions range from carbon starvation to cytochrome oxidase and to putative cytoplasmic proteins. Despite the diversity of gene annotations, their regulation is dominated by NsrR, FNR and Fur.

Table 14: The genes of the core NO regulon, expression levels and their regulation

		Expression levels: Endogenous NO			
Gene	**Gene product**	**5 h to 80 h**	**5 h to 120 h**	**Exogenous NO:**	**Regulation**
cstA	Carbon starvation protein	7	6	4	**CsrA** (Dubey et al, 2003) **σ^{70}** (Blum et al, 1990) **cAMP** (Schultz et al, 1988; Schultz & Matin, 1991)
***cydB* (*cyd* operon)**	Cytochrome *d* terminal oxidase polypeptide subunit II	13	10	4	**FNR** (Govantes et al, 2000)
hcr	NADH oxidoreductase for *hcp* gene product	77	67	2	**NsrR** (Filenko et al, 2007; Filenko et al, 2005; Rodionov et al, 2005) **FNR** (Constantinidou et al, 2006) **OxyR** (Almeida et al, 2006; Seth et al, 2012)
hmpA	Flavohaemoglobin with nitric oxide di-oxygenase and nitric oxide reductase activity	172	121	185	**MetR** (De Groote et al, 1996) **NsrR** (Bang et al, 2006; Karlinsey et al, 2012) **FNR** (Cruz-Ramos et al, 2002; Poole et al, 1996) **Fur** (Crawford & Goldberg, 1998a; Hernández-Urzúa et al, 2007)
***norV* (*nor* operon)**	Flavorubredoxin-type nitric oxide reductase	8	4	613	**NorR** (Gardner et al, 2003; Gardner et al, 2002; Hutchings et al, 2002; Mukhopadhyay et al, 2004) **NsrR** (Partridge et al, 2009)
yeaR-yoaG	Putative cytoplasmic protein with DUF1971 for tellurite resistance; putative cytoplasmic protein	30; 10	41; 12	27; 7	**NarL** (Constantinidou et al, 2006; Lin et al, 2007; Squire et al, 2009) **NsrR** (Filenko et al, 2007; Karlinsey et al, 2012)
ygbA	Putative cytoplasmic protein	11	12	14	**NsrR** (Bodenmiller & Spiro, 2006; Filenko et al, 2007; Gilberthorpe et al, 2007; Karlinsey et al, 2012; Rodionov et al, 2005)
ytfE	[Fe-S] cluster repair protein	43	36	137	**FNR** (Constantinidou et al, 2006) **NsrR** (Bodenmiller & Spiro, 2006; Karlinsey et al, 2012; Rodionov et al, 2005)

In the next sections, these genes are introduced further and the information available in the literature is discussed.

3.5.1 cstA

CstA has been annotated as a carbon starvation protein. It has been identified as a potential novel *Salmonella* virulence factor using a *Caenorhabditis elegans* infection model (Tenor et al, 2004). In *E. coli cstA* has been described as a peptide transporter (Schultz et al, 1988; Schultz & Matin, 1991). In UniProtKB/Swiss-Prot (accession number P15078*)* CstA has been annotated to contain eighteen trans-membrane domains, supporting the indication that it is an integral membrane protein. Transcription of *cstA* in *E. coli* is induced mainly during carbon starvation, but also occurs under nitrogen limitation (Dubey et al, 2003; Schultz et al, 1988). Transcription in *E. coli* has been determined to be σ^{70}-dependent, but a role of CRP-cAMP for regulation has also been identified (Blum et al, 1990; Schultz et al, 1988; Schultz & Matin, 1991). CstA is also regulated at the translational level by the carbon storage regulator protein CsrA, which blocks the Shine-Delgarno sequence upstream of *cstA* (Dubey et al, 2003). No previous role in NO detoxification or damage repair has been recorded in the literature for CstA.

3.5.2 cydB

CydB has been annotated as a polypeptide subunit II of the cytochrome *d* terminal oxidase containing three haem cofactors (Osborne & Gennis, 1999). The structural analysis of CydAB has revealed the periplasmic location of a ‘Q loop’ implicated in quinol binding (Zhang et al, 2004). Hence, it has been proposed that CydAB has a function in quinol oxidation leading to the production of a proton motive force as part of the electron transport chain. A low NO-reductase activity has previously been reported, which is attributed to the shared phylogenesis with e.g. haem-containing NO reductases from *B. japonicum* (Saraste & Castresana, 1994). Measurements using purified cytochrome *bd* of *E. coli* has revealed no measurable NO reductase activity (Borisov et al, 2004). Evidence that NO inhibits enzyme activity even during low oxygen concentrations has led to the conclusion that *E. coli* CydAB is not functioning as a NO reductase during infection conditions. Such inactivation by NO has been shown to be reversible and quick recovery occurs. sIn *E. coli*, it

has been shown that *cydB*, together with *cyoA*, encodes for a cytochrome *bo* terminal oxidase in aerobic conditions and for a cytochrome *bd* type in microaerophilic conditions (Puustinen et al, 1991). Recently, a correlation between the regulation of *cyoA* and *cydB* has been shown: The deletion of one gene results in significant increase in expression of the respective other gene (Kumar & Shimizu, 2011). In 2000, Govantes *et al.* have investigated the regulation of the *cydAB* operon and have determined the interplay of the five promoters with regulatory factors (Govantes et al, 2000). The regulation of promoters P1 to P4 is mediated by the presence of oxygen as well as FNR and ArcA, part of a two-component system together with ArcB. Without the presence of a functional ArcA, no repression by FNR is observed and the mode of action is described as anti-activating. Promoter P5 has been found to be less tightly regulated and no effect of FNR- or ArcA-binding has been found. Increased transcription levels of *cydAB* are identified in *E. coli* when cells are grown anaerobically in minimal salts medium and exposed to a NO donor substance (Filenko et al, 2007). It has been shown that bacterial respiration is disrupted in the presence of NO (Stevanin et al, 2000). An increased amount of cytochrome oxidases could be a mechanism to ensure that a sufficient amount of oxidases are present to allow successful respiration by supplying a stable proton motive force across the membrane (Giuffre et al, 2012). Changes in expression levels of *cydAB* have been recorded in a study involving *M. tuberculosis* during the transition from acute to chronic infection of mice tissue (Shi et al, 2005). These changes are attributed to a change in aerobic respiration that allows *M. tuberculosis* to adapt to the host immune system. The multiplication rate and persistence of *S*. Typhimurium Δ*cydA* mutants are significantly reduced in chicken macrophages and in the reticulo-endothelial system of chicken (Turner et al, 2003). In a murine macrophage experiment, this phenotype is reversed and increased persistence is recorded. In addition, the mutant shows better adaptation to low pH. Differences between these phenotypes are attributed to the use of different serovars throughout the study. The findings of intracellular survival assays using Δ*cydA* mutants support a role of CydAB during infection through defence from NO-mediated stress and adaptation to environments of low oxygen concentrations.

CydB expression has been found to be increased after exposure to NO or in intracellular environments, indicating a role of CydB during infection.

3.5.3 hcr

Hcr belongs to one operon with *hcp* and encodes for a NADH-dependent oxidoreductase that modifies the gene product of *hcp*, a hydroxylamine reductase gene. Different studies have proposed different possible functions for this operon in *E. coli:* Wolfe *et al.* have shown that they function as a hydroxylamine reductase and Almeida *et al.* have shown peroxidase activity (Almeida et al, 2006; Wolfe et al, 2002). After a NsrR-binding site has been predicted (Rodionov et al, 2005), the binding site and its regulation by NsrR have been confirmed (Filenko et al, 2007; Filenko et al, 2005). The previously proposed induction by hydroxylamine, however, has not been confirmed (Filenko et al, 2007). Despite the proven repression of NsrR on the expression of *hcr*, it has been shown that it also belongs to the FNR regulon (Constantinidou et al, 2006; Kim et al, 2003). Another regulator of *hcp-hcr* is OxyR and an induction of gene expression by hydrogen peroxide has been demonstrated (Almeida et al, 2006). OxyR is a transcriptional regulator, which responds to the presence of hydrogen peroxide and consequently it has been shown to regulate genes in response to oxidative stress (Zheng et al, 1999). In addition, the binding of OxyR to the *hcp promoter* has been shown recently (Seth et al, 2012). The tight regulation by NsrR has been shown to be synonymous to NsrR regulation of *hmpA* and *ytfE* (Filenko et al, 2007) and an increased expression of *hcp* has also been noted after the addition of 200 µM GSNO to a continuous culture of *E. coli* (Flatley et al, 2005). Recent experiments have confirmed the regulation of *hcp-hcr* by NsrR in *Salmonella* using a microarray approach (Karlinsey et al, 2012). However, no change in NO sensitivity has been observed for a *hcp* deletion mutant strain in comparison to the wild-type phenotype, although a role for Hcp-Hcr has been proposed for NO detoxification and resistance against NO-mediated inhibition of respiration.

Induction of *hcp-hcr* has also been observed within activated RAW264.7 macrophages using *gfp*-fusions (Kim et al, 2003). Furthermore, deletion of *hcp-hcr* results in a lower LD_{50} than wild-type when low inoculation doses are used.

No differences in intracellular survival and replication are observed at high doses. This contradicts findings that a deletion strain of *hcp* outcompetes wild-type cells for survival in the spleen of mice (Karlinsey et al, 2012). These findings highlight that the precise roles of *hcr* and *hcp* have yet to be identified and that more research is needed to identify their role during infection.

3.5.4 hmpA

HmpA is one of the three identified NO detoxification genes and has been discussed in detail in Section 1.6.1.

3.5.5 norV

The gene *norV* encodes a flavorubredoxin-type NO reductase and is one of the characterized NO detoxification enzymes of *Salmonella*. Its protein function and role during infection have been discussed in Section 1.6.2.

3.5.6 yeaR-yoaG

YeaR and *yoaG* are two of the many yet uncharacterized genes from the *Salmonella* genome and belong to one operon. *YoaG* contains a domain of unknown function (DUF1869) that has not been characterized further.

YeaR also contains a domain of unknown function (DUF1971) and it belongs to a family of uncharacterised domains predominantly found in tellurite resistance proteins (Marchler-Bauer et al, 2009). This domain has been found in a range of other bacteria, most of which belong to the Gamma-proteobacteria, ranging from soil-dwelling organisms such as *P. denitrificans* to pathogens like *Yersinia pestis* and *H. influenzae* or gastrointestinal inhabitants, e.g. *E. coli* K-12. This DUF is also found in the *S*. Typhimurium-specific gene *STM1808*, which will be discussed later.

Irrespective of its putative role in tellurite resistance, it has been reported that the expression of *yeaR* in *E. coli* is induced by nitrate (Constantinidou et al, 2006) as well as by the presence of nitric oxide (Justino et al, 2005). Further studies in *E. coli* have also determined that *yeaR* expression is repressed by

NsrR (Filenko et al, 2007; Partridge et al, 2009) and by FNR, although no FNR site has been identified (Constantinidou et al, 2006). Evidence for NsrR regulation in *Salmonella* has been published recently (Karlinsey et al, 2012). Lin *et al.*, though, have shown that regulation of *yeaR* and its downstream neighbour *yoaG* occurs in a nitrate- and nitrite-dependent manner under the control of nitrate/nitrate response regulator NarL as well as NsrR with no involvement of FNR (Lin et al, 2007). This is further supported by biochemical analyses showing that NarL binds to a binding site upstream of *yeaR* without a requirement for FNR (Squire et al, 2009). De-repression of NsrR, however, is necessary before transcription of *yeaR-yoaG* is initiated. In addition, Fis, a sequence-specific DNA-binding protein, interferes with NarL binding. Two Fis-sites close to the DNA-binding site of NarL lead to displacement of NarL by Fis, which prevents formation of an open complex that would initiate transcription of the downstream operon.
The influence of nitrate, nitrite and NsrR on transcription indicates that YeaR might play a role during nitrosative stress and this link needs to be investigated further.

YoaG and *yeaR* are part of one operon under the regulation of NarL and repression of NsrR (Constantinidou et al, 2006; Filenko et al, 2007; Lin et al, 2007). In *S*. Typhimurium, a putative nitric oxide reductase (*STM1273*) is found downstream of the *yeaR-yoaG* operon. In 2005, the Northeast Structural Genomics Consortium (NESG) added a solution structure to the Protein Knowledgebase (UniProtKB/Swiss-Prot, accession number P64496) with regards to the structure of the *E. coli* MG1655 *yoaG* gene and revealed the presence of two β-strands and two α-helices. However, no functional role has been determined.

3.5.7 ygbA

Functionally and structurally, not much is known about YgbA. *YgbA* expression is induced by the addition of GSNO and sodium nitrite (Mukhopadhyay et al, 2004) and a computationally predicted NsrR-site has been found upstream (Rodionov et al, 2005) which has subsequently been experimentally confirmed

in *E. coli* (Bodenmiller & Spiro, 2006; Filenko et al, 2007). and *S.* Typhimurium (Gilberthorpe et al, 2007). In the *S*. Typhimurium genome, *ygbA* is neighboured by operons *hycDCBA*, *hypABCDE* and *sitABCD* encoding for hydrogenases and manganese/iron transport systems, respectively. The reactivity of NO with [Fe-S] clusters and the subsequent release of free iron results in the need for the cell to sequester iron to reduce its reactivity and to allow its transport. For this reason, the proximity of *ygbA* to operons encoding iron transport systems provides a potential link to its involvement during nitrosative stress.

3.5.8 ytfE

YtfE is also known as *nipC* and RIC in the literature (Kim et al, 2003; Overton et al, 2008). Neither structure nor function have been determined in *Salmonella* so far, but extensive research has been conducted using *E. coli*. Initial studies have identified nitrite and GSNO as potential inducers of gene expression (Kim et al, 2003; Mukhopadhyay et al, 2004). The identification of a NsrR binding site has followed, first computationally (Rodionov et al, 2005) and later also experimentally (Bodenmiller & Spiro, 2006). Additionally, the strong induction of gene expression by nitrate and nitrite reported in 2006 (Constantinidou et al, 2006) has been supported by findings *that ytfE* expression is activated by NarL (Filenko et al, 2007). Justino *et al.* have been able to assign YtfE a role in the biogenesis of iron-sulphur clusters in *E. coli* (Justino et al, 2006). Furthermore, it has been determined that YtfE contains an iron-sulphur cluster itself, forming a homodimer (Todorovic et al, 2008). The protection against oxidative stress has been proven in another study published in 2007 (Justino et al, 2007). Both Justino *et al.* and Pullan *et al.* describe a significant increase in gene expression when NO is added during aerobic growth (Justino et al, 2005; Pullan et al, 2007). When *ytfE* is deleted from the *S*. Typhimurium genome, the deletion mutant does not show a higher susceptibility towards NO, using GSNO as an NO-donor, in comparison to the wild-type phenotype (Gilberthorpe et al, 2007). Moreover, the deletion has been reported to increase the persistence of the strain during the infection of mice (Kim et al, 2003). Since [Fe-S] clusters are especially at risk during nitrosative stress, it would be expected to see an

increased expression of proteins like YtfE that are able to counteract the disassembly of [Fe-S] clusters caused by NO.

Despite the knowledge that *ytfE* is induced by nitrite under anaerobic conditions (Kim et al, 2003), it has been found that a *ytfE* mutant strain is significantly more virulent after intraperitoneal infection of mice than the isogenic parent strain (Rowley *et al.*, unpublished data). Consequently, more extensive work has to be performed to fully understand the role of YtfE in *Salmonella* especially during the infection process.

3.6 Discussion

3.6.1 Expression changes to endogenous NO

Exposure to endogenous NO has increased the levels of mRNA of 46 genes significantly. A list of the ten most highly induced genes is shown in Table 15, highlighting that at both 80 and 120 hours of continuous growth and NO exposure, five *nar* operon genes are highly induced. *NarG* tops the list for both rankings and indicates that the encoded nitrate reductase is the primary enzyme needed during anaerobic respiration and the use of nitrate as an electron acceptor. The level of *hmpA* are second highest, supporting the need for a NO detoxification mechanism during endogenous NO exposure.

Other genes previously shown to play a role during NO defence are also highly up-regulated, e.g. encoding for the [Fe-S] repair protein YtfE. The precise role of the gene products of *hcr* and *yeaR* has not been determined, but six and five other studies, respectively, have also seen increased gene expression under NO stress conditions. In addition, the outer membrane protein precursor gene *ompF* shows increased expression for the 80 hour time point. NO exposure affects the integrity of the cell wall and increased expression of genes such as *ompF* would help to diminish the damage that endogenous NO causes to the cell wall proteins. For the 120 hour time point, the formate dehydrogenase-N subunit alpha-encoding gene *fdnG* is also highly induced. This reflects the need for *S*. Typhimurium to keep its metabolism running; even if endogenous NO concentrations are unfavourable for growth.

Table 15: Top Ten of most highly induced genes after 80 and 120 hours of continuous anaerobic growth of *S*. Typhimurium SL1344.

Only genes with increased expression levels are shown for comparison.

5 h to 80 h			**5 h to 120 h**		
	Gene:	**Fold change:**		**Gene:**	**Fold change:**
1	*narG*	203	**1**	*narG*	179
2	*hmpA*	172	**2**	*hmpA*	121
3	*narJ*	80	**3**	*narJ*	82
4	*hcr*	77	**4**	*hcr*	67
5	*narK*	73	**5**	*narK*	57
6	*narH*	54	**6**	*narH*	50
7	*ytfE*	43	**7**	*yeaR*	41
8	*yeaR*	30	**8**	*ytfE*	36
9	*narI*	26	**9**	*narI*	25
10	*ompF*	23	**10**	*fdnG*	21

All in all, the genes that are most highly induced reflect the growth conditions faced by *S*. Typhimurium during continuous anaerobic growth and during NO production as a by-product of its metabolism. *NarGHIJK* and *fdnG* support anaerobic respiration, whereas *hcr*, *hmpA*, *ompF*, *yeaR* and *ytfE* provide protection against NO damage through detoxification and damage repair.

3.6.2 Expression changes to exogenous NO

Addition of exogenous NO requires a faster shift in gene expression in order to be able to survive the sudden exposure to NO in comparison to the depletion of oxygen and the gradual use of nitrate in anaerobic respiration that results in the formation of endogenous NO. The response to the NO addition manifests itself in the significant increase of expression levels of 139 genes. The ten genes with the highest expression changes have been listed in Table 16. The high levels of *norVW* and *hmpA* clearly point out the need for rapid NO detoxification. Furthermore, *ytfE* is on the list that has been shown to support NO-caused damage repair.

The role of STM1808, containing a domain of unknown function implicated in tellurite resistance; STM1250, a putative cytoplasmic protein and of STM1868A,

encoding for a lytic enzyme, in the context of NO detoxification or damage repair are not yet clear. The need to reduce the amount of free iron within the cell that would result from damage to [Fe-S] clusters becomes evident with the up-regulation of *sitABC*.

Again, the results reflect the changes in environmental conditions that *S*. Typhimurium faces during the experiment. NO interacts with [Fe-S] of proteins, releasing free iron into the cell. In order to prevent these Fe ions to fuel the Fenton reaction, leading to an increase in ROS to be formed, iron regulatory proteins are needed, encoded by the *sit* operon. Additionally, the protein repair is supported by increasing the expression of *ytfE*. Lastly, NO itself is detoxified via the increase in expression of *hmpA* and *norVW*. The exact role of *STM1250*, *STM1808* and *STM1868A*, all *Salmonella*-specific genes, yet remains unclear. Only the increased expression of *STM1808* and NO sensitivity of a respective deletion strain have been reported, but a further characterisation of STM1808 function is still outstanding (Karlinsey et al, 2012).

Table 16: Top Ten of most highly induced genes after addition of 40 µM NO to anaerobically growing *S*. Typhimurium SL1344

	Gene:	**Fold change:**
1	*norV*	613
2	*norW*	204
3	*STM1808*	195
4	*hmpA*	185
5	*STM1868A*	169
6	*ytfE*	137
7	*sitB*	94
8	*sitA*	88
9	*STM1250*	81
10	*sitC*	80

3.6.3 Core NO regulon

Based on the results of both microarray data sets, it has been possible to identify nine genes that are up-regulated after both conditions of NO exposure and that potentially are important for NO detoxification. Of these, *hmpA*, *norV* and *ytfE* already have been shown to aid in NO defence. Other genes have, so far, not been shown to be involved or only recently have been suggested to play a role. Together with genes that show homology to the core NO regulon set, it remains to be determined what their contribution and potential function during NO detoxification is. Therefore, deletion mutant strains are constructed in the genes of the core NO regulon as well as of other genes such as *STM1808*, which share homology with core regulon genes. The aim is to allow the testing of these strains under nitrosative stress conditions. In order to validate potential phenotypes, complementation constructs are also made.

4 Contribution of NO induced genes to *Salmonella* NO resistance

4.1 Introduction

NO is a highly reactive gas with a short half-life time. It rapidly reacts with oxygen to form nitrogen dioxide. It is highly toxic for humans and hence in gas form cannot be readily used for experimental procedures. A range of compounds that act as NO donors and release NO under certain environmental conditions as well as pure NO are available and have been extensively used in the literature. NONOates are a family of compounds with two alkyl groups and several sequential nitrogen atoms. Although all of the family members can release NO upon contact with water or when the pH of a solution is lowered, half-life times of NO release vary significantly between the different NONOates. Half-lives of these compounds are as fast as 1.8 seconds for Proline-NONOate (Cruz-Ramos et al, 2002) and are as slow as 20 hours for DETA-NONOate (Jones-Carson et al, 2012). These differences in half life afford the choice of NO donor according to experimental requirements, as each compound has a certain ratio of NONOate concentration required to release a specific concentration of NO that needs to be taken into consideration: The addition of 1 µM of Proline- or Spermine-NONOate results in the release of 2 µM NO and adding 1 µM of diethylamine NONOate releases 1.5 µM NO. DETA-NONOate releases 6 µM of NO with the addition of 1 mg x mL^{-1}. For example, reports of their use in *E. coli* and *Salmonella* experiments show the use of different concentrations of NONOate, covering a range of 50 µM (Baptista et al, 2012) to several mM of NO released (Karlinsey et al, 2012). In addition, a combination of different compounds is used to create a more steady NO release (Pullan et al, 2008).

S-nitrosothiols such as *S*-nitrosoglutathione (GSNO) can also be used as NO donor compounds. The activity of GSNO is not oxygen dependent and achieves the highest antibacterial activity against stationary cells (De Groote et al, 1996). It is easily used for disk diffusion assays where GSNO is applied onto a disk on top of an inoculated agar plate (De Groote et al, 1996; Eriksson et al, 2000). It has been reported that even high concentrations of GSNO [500 mM] only exert a cytostatic effect on *S*. Typhimurium. The release of NO by GSNO is the result of a complex line of reactions, which result in the release of other by-products such as thiol radicals (Singh et al, 1996). Thus, although GSNO has practical advantages for laboratory use in comparison with the more expensive NONOates, the interference of thiol by-products in any phenotypes observed

cannot be excluded. Differences in sensitivity towards GSNO and other NO donors have been observed. A Δ*metL* mutant strain of *S*. Typhimurium is only sensitive towards GSNO, but shows no signs of increased sensitivity towards the NO donor DETA-NO (De Groote et al, 1996). This highlights the importance of cross-checking any NO sensitivity observed with GSNO by using an alternative NO source to exclude interference of thiols.

Instead of using NO-generating compounds, a saturated NO solution has been generated. Although this saturated NO solution is only stable for 24 hours and reacts with oxygen, it allows a more defined administration of NO to liquid growth media. For an analysis of changes in anaerobic growth by NO, the use of such a saturated NO solution is the most suitable as no other RNS are generated during the preparation of the solution.

Anaerobic NO detoxification is essential for the survival of *Salmonella* during infection. Several of the core NO regulon genes have already been shown to be of importance. The enzymatic NO detoxification of HmpA and NorV has been demonstrated and during anaerobic conditions, NorV has been shown to contribute more to NO defence (Mills et al, 2008). Deletion of *ytfE* does not increase the aerobic NO sensitivity, but shows decreased survival during mice infection (Gilberthorpe et al, 2007; Kim et al, 2003). The other genes have not yet been investigated for their anaerobic NO sensitivity, but have not shown any increased sensitivity under aerobic conditions. Similar to other related NsrR-regulated genes, such as *STM1808* (discussed in Chapter 5) and *tehB*, they possibly contribute to anaerobic NO detoxification.

4.2 Aim

The aim of this chapter is to determine the contribution of the core NO regulon, as identified in Chapter 3, as well as the remaining NsrR regulated genes to anaerobic detoxification of NO. In doing this, we experimentally confirm NsrR regulation of the *Salmonella*-specific gene *STM1808*, whose function is unknown.

4.3 Methods

4.3.1 β-galactosidase assay

For the investigation of NsrR-regulation of *STM1808*, *lacZ* fusion strains have been designed and β-galactosidase activity is measured as described in 2.10. The enzyme β-galactosidase cleaves the substrate ONPG, resulting in the generation of the yellow product *o*-nitrophenyl. The product *o*-nitrophenol absorbs light at 420nm and any changes in *o*-nitrophenol concentration are measured using a spectrophotometer.

4.3.2 Mutant construction

As described in Chapter 3, expression of several genes is stimulated by the addition of NO. For further investigation of anaerobic NO sensitivity, the core NO regulon genes are chosen as they have increased expression levels during both endogenous and exogenous NO exposure. In addition, the related genes *STM1808*, *tehA* and *tehB* are chosen due to their homology to the NsrR regulated gene, *yeaR*, and their up-regulation after exogenous NO exposure.

Single deletion mutant strains have been constructed using the *de novo* mutagenesis method described by Datsenko & Wanner in 2000 (refer to 2.4 for the protocol). To avoid further recombination events, the mutation is transferred into a clean parent strain SL1344 using P22 bacteriophage transduction (2.6). The replacement of the gene of interest with an antibiotic gene cassette is confirmed using colony PCR and visualized on an agarose gel according to the protocols describe in 2.5. The images of gel electrophoresis after PCR amplification using external primers are shown in Figure 12.

Figure 12: Gel electrophoresis images of the PCR confirmation of deletion mutations.
The predicted band sizes for SL1344 wild-type genes and deletion mutant strains are as follows:
A) SL1344 *cstA*: 2,151 bp, Δ*cstA*: 1,300 bp;
B) SL1344 *cydB*: 1.140 bp, Δ*cydB*: 1,700 bp; SL1344 *STM1273*: 642 + 320* bp, Δ*STM1273*: 1,700 bp;
C) SL1344 *hcr*: 972 + 320* bp, Δ*hcr*: 1,700 bp;
D) SL1344 *STM1808*: 350 bp, Δ*STM1808*: 1,300 bp;
E) SL1344 *tehA*: 1,050 bp, Δ*tehA*: 1,300 bp;
F) SL1344 *ygbA*: 345 bp, Δ*ygbA*: 1,300 bp.
Asterisk indicates the use of external primers that are further up- and downstream of the gene of interest, therefore creating larger bands as up to 320 bp are added to the amplification product.

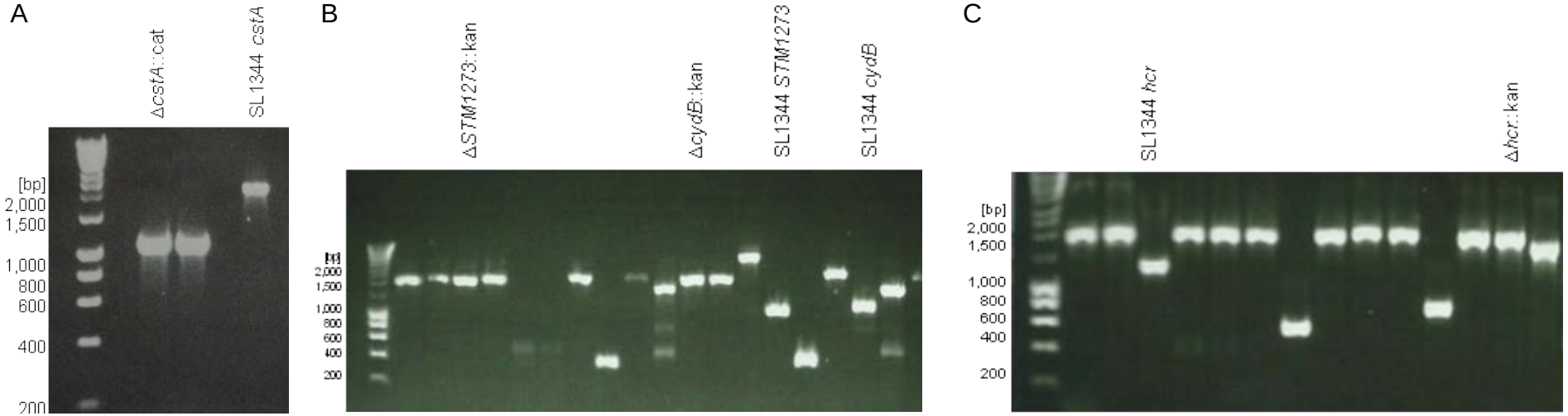
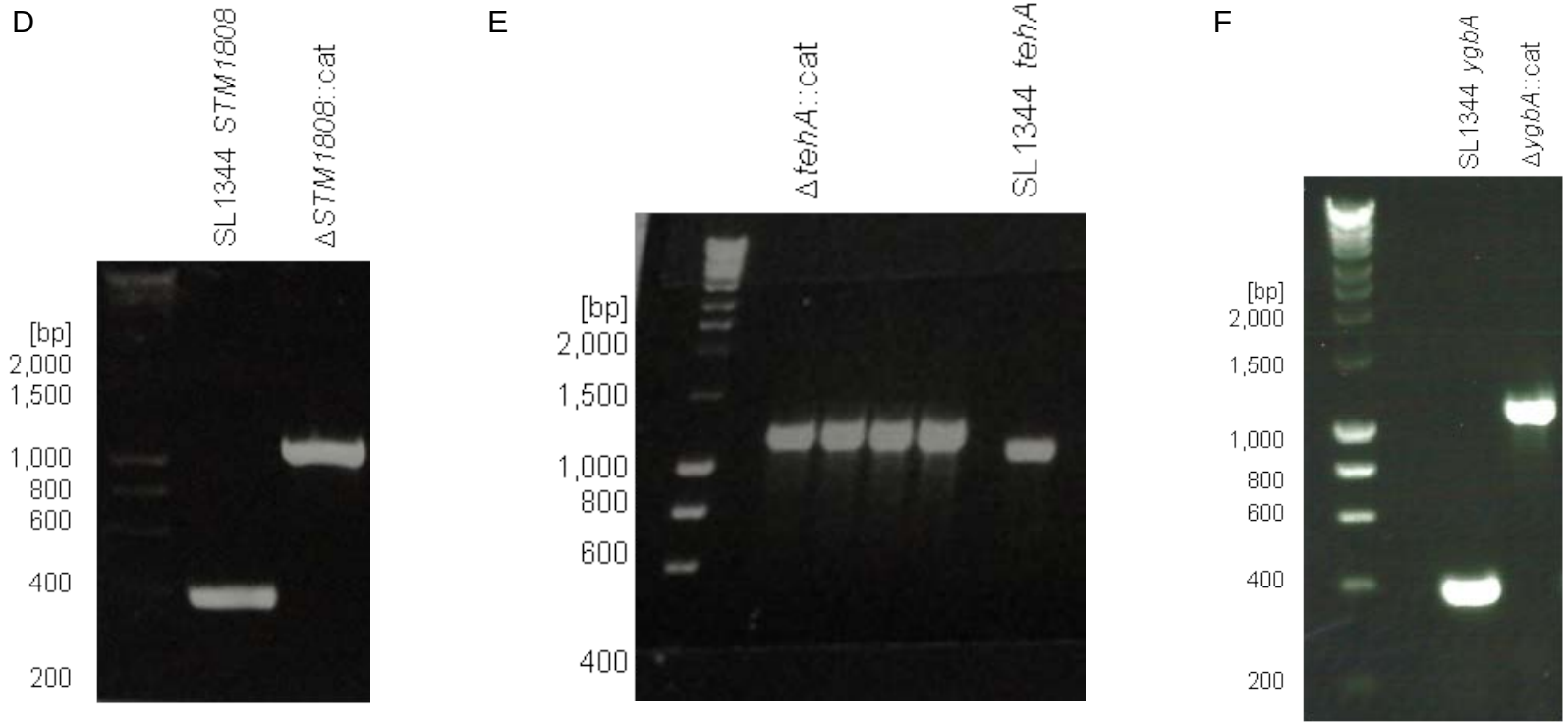


Figure 12 continued: Gel electrophoresis images of the PCR confirmation of deletion mutations.
The predicted band sizes for SL1344 wild-type genes and deletion mutant strains are as follows:
A) SL1344 *cstA*: 2,151 bp, Δ*cstA*: 1,300 bp;
B) SL1344 *cydB*: 1.140 bp, Δ*cydB*: 1,700 bp; SL1344 *STM1273*: 642 + 320* bp, Δ*STM1273*: 1,700 bp;
C) SL1344 *hcr*: 972 + 320* bp, Δ*hcr*: 1,700 bp;
D) SL1344 *STM1808*: 350 bp, Δ*STM1808*: 1,300 bp;
E) SL1344 *tehA*: 1,050 bp, Δ*tehA*: 1,300 bp;
F) SL1344 *ygbA*: 345 bp, Δ*ygbA*: 1,300 bp.
Asterisk indicates the use of external primers that are further up- and downstream of the gene of interest, therefore creating larger bands as up to 320 bp are added to the amplification product.



4.3.3 Anaerobic growth

To achieve anaerobic conditions, the cultures are grown in Hungate tubes and oxygen-free nitrogen is used to remove residual oxygen in the head space of the tubes. The medium used is a minimal glucose medium instead of the minimal salts medium used previously (Mills et al, 2008). This eliminates fluctuations in growth and results in higher optical densities of cultures throughout the experiment. In turn this allows more reproducible datasets to determine the effect of NO addition on growth. In accordance with previous reports, a concentration of 40 µM aqueous NO similar to the NO concentrations in the SCV is used for anaerobic experiments (Mills et al, 2008; Vazquez-Torres et al, 2000a). These experimental conditions reflect the NO concentration as well as scarcity of nutrients and oxygen available to *Salmonella* within the SCV (Eriksson et al, 2000; Eriksson et al, 2003).

The strains are grown as outlined in 2.9.3. The addition of 40 µM NO is performed once the growth of the culture reaches an absorbance of 0.1. Subsequently growth is monitored at 30-minute intervals for up to 8 hours afterwards. NO addition is indicated by the arrow in Figure 14-16. A saturated NO solution is produced by scrubbing NO gas with oxygen-free NaOH solution. As such, the gas is used to saturate a volume of distilled water with a pH of 3 (Baptista et al, 2012; Mills et al, 2008). NO retains its reactivity with oxygen and therefore the NO solution needs to be used within 24 hours. Using a Hamilton syringe, addition of precise NO concentrations to anaerobic cultures is achieved.

The impact of 40 µM aqueous NO to the growth rate of anaerobic cultures is determined for several groups of genes: The first group comprises mutant strains where genes involved in NO detoxification, either known (*hmpA*, *nrfA*, *norV, ytfE*) or predicted (*STM1273*), and NO-mediated regulation (*nsrR*) are investigated. Next, mutant strains of the core NO regulon are assayed. The third group contains deletion strains of genes implicated for tellurite resistance.

4.4 NsrR regulation of *STM1808*

Computational analysis of the S. Typhimurium genome has previously shown a NsrR-binding site located in the promoter of the *Salmonella*-specific gene *STM1808* therefore suggesting an involvement in NO defence (Rodionov et al, 2005). Furthermore, the results shown in 3.4.2 highlight that *STM1808* expression levels increases by 195 fold during the exposure to exogenous NO. This fold increase is very similar to *hmpA* levels (185), which is regulated by NsrR. In order to establish whether *STM1808* indeed is regulated by NsrR, a *lacZ* transcriptional fusion which contained the suspected NsrR-binding site upstream of *STM1808* is created.

In Figure 13, the results of the β-galactosidase assay can be seen. In the wild-type parent strain, the expression of β-galactosidase is repressed. The level of *o*-nitrophenol indicative of the amount of β-galactosidase present is at an equally low level to the levels of cells from the wild-type and of the *nsrR* deletion strain transformed with empty plasmid.

In the Δ*nsrR* strain the activity of the *STM1808* promoter fusion is increased to one hundred fold. This indicates that loss of NsrR results in de-repression of *STM1808* and experimentally confirms for the first time that NsrR acts as a repressor of *STM1808* expression. In support of the array data in Chapter 3, this also further supports a role for *STM1808* in NO detoxification. As a result of this, *STM1808* and the other tellurite resistance genes are included in this chapter to determine their role in *Salmonella* NO resistance.

4.5 Anaerobic growth in the presence of 40 µM NO

4.5.1 Sensitivity of strains with deletions of known NO detoxification genes

To confirm the validity of our experimental set-up, we have first analysed the affect of NO addition to the to the growth rates of strains carrying mutation in genes previously implicated in *Salmonella* NO detoxification. The addition of 40 µM NO to the wild-type strain SL1344 results in growth arrest of

approximately one hour (Figure 14). The levels of sensitivity for Δ*norV* and Δ*nrfA* match previously recorded phenotypes (Mills et al, 2008). Deleting *norV* results in a more pronounced sensitivity than deleting *nrfA*, but both deletion strains are more susceptible to growth delay by NO than the wild-type. The growth rate of Δ*nrfA* drops by half and Δ*norV* grows at a rate of around one third of the rate displayed in the absence of NO. This confirms their attributed role of nitric oxide reduction under anaerobic conditions.

No significant difference in growth arrest can be observed for the deletion strains of *hmpA*, *STM1273* and *ytfE*.

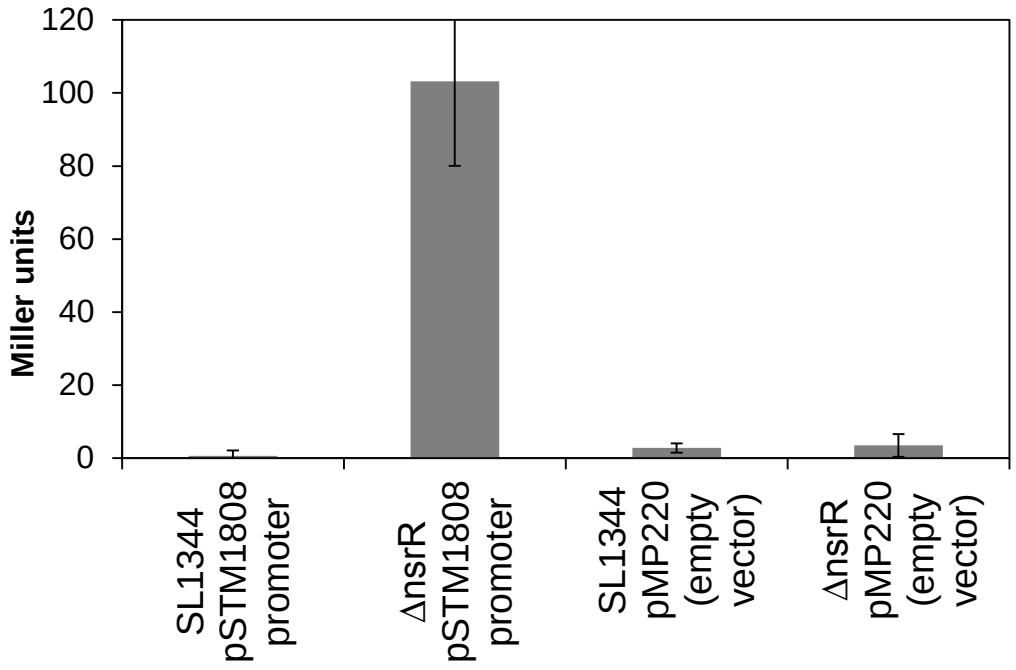


Figure 13: β-galactosidase activity of the SL1344 parent and the Δ*nsrR* strains. β-galactosidase activity of the SL1344 parent and the Δ*nsrR* strains transformed with pMP220 containing the *STM1808* promoter (p*STM1808* promoter) or the empty plasmid (pMP220) is presented. The results show the mean of three biological replicates with standard deviations.

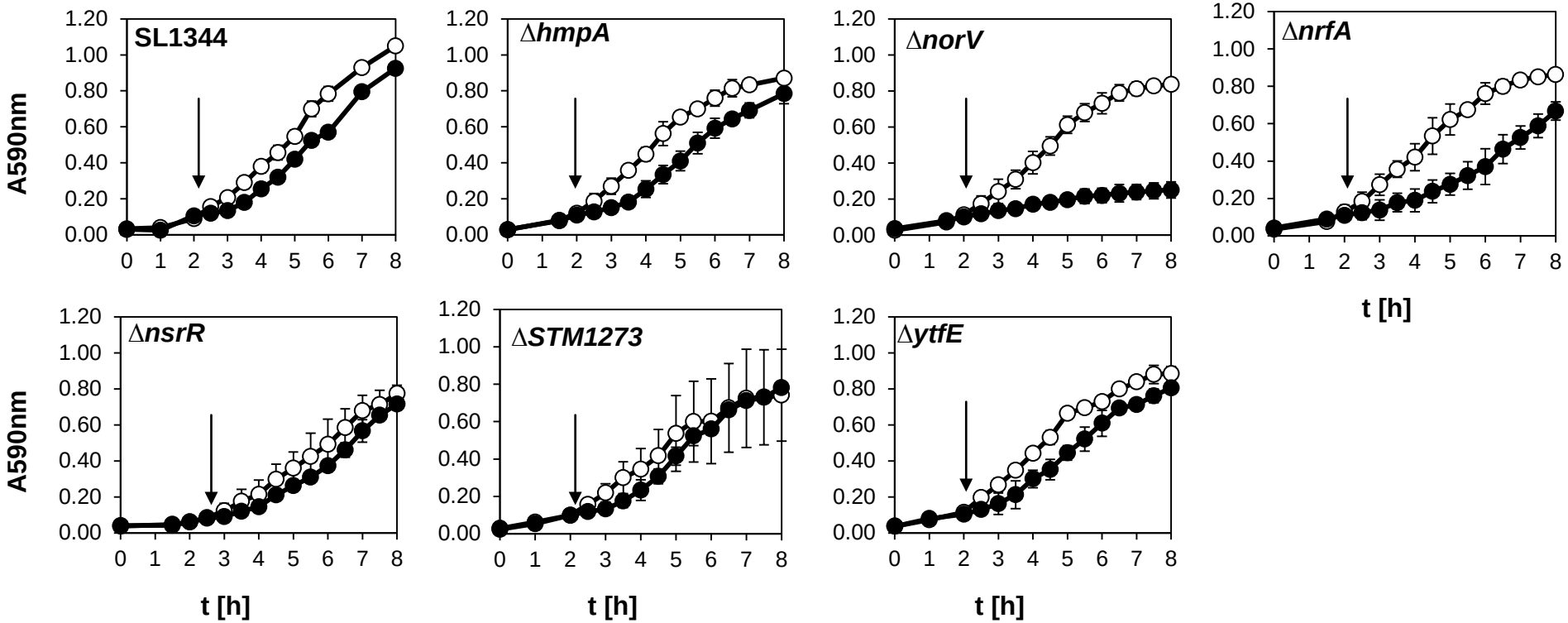


Figure 14: Anaerobic growth of NO detoxification mutant strains in MGM (0.05% (w/v) casamino acids) in the absence and presence of 40 µM NO.

Cultures have been grown in MGM anaerobically in Hungate tubes at 37°C. Black arrow: Once an absorption value of 0.1 is reached, 40 µM aq. NO is added to the cultures (black circles) or no addition is performed (white circles). Mean and standard deviation of three biological replicates are shown for each strain.

The minor contribution of HmpA for anaerobic NO detoxification has been shown previously (Mills et al, 2008) and corresponds to the findings shown here. The annotation for *STM1273* does not indicate whether the enzyme would perform nitric oxide reductase activity anaerobically or aerobically. The analysis of the growth rates with and without the addition of NO show that there are no significant differences of Δ*ytfE* and Δ*STM1273* growth in comparison to the respective wild-type growth rates (Table 17): The addition of NO results in changes in the growth rates in comparison to the growth rate in the absence of NO, but these changes are not significantly different from the growth rates measured for SL1344. It has been shown here that no significant anaerobic activity is noted. In other studies, no increased NO sensitivity for a *Salmonella ytfE* deletion strain has been observed, which correlates with our results (Gilberthorpe et al, 2007).

The deletion of *nsrR* resulted in a phenotype more resistant towards NO addition, causing only a minor growth arrest in the growth of the culture. The growth rate after NO addition displays only a minor reduction when compared to its rate without nitric oxide.

Statistical analysis has highlighted that the deletion strain Δ*nsrR* grows better in the presence of NO than the wild-type. Previous studies have reported similar findings (Gilberthorpe et al, 2007; Karlinsey et al, 2012): Removing NsrR from the pool of regulators in the cell results in the de-repression of NO detoxifying genes, allowing their expression to be continuous, irrespective of the presence of NO. As a result, it has been shown that a *nsrR* deletion strain is not affected by the presence of NO (Gilberthorpe et al, 2007; Karlinsey et al, 2012). The results shown here further accentuate this finding.

4.5.2 Sensitivity of strains with deletions in the core NO regulon

The next group of mutant strains comprises single deletion mutant strains from the list of the core NO regulon. All these genes are significantly increased in expression after NO addition to growing cultures or during conditions favouring endogenous NO production, indicating a role in coping with NO-caused stress (Chapter 3).

Table 17: Growth rates of wild-type and NO detoxification deletion strains from the time of NO addition until two or three hours post addition, respectively.

The results are the mean of a minimum of three biological and two technical replicates with their standard deviations. Student t tests were performed and all growth rates with NO are significantly lower in comparison to growth rates without nitric oxide for each strain. Significant differences to corresponding wild-type growth rates are indicated by grey colouration.

Strain	**NO**	**Growth rate ± st. dev.**	
		2 h	**3 h**
SL1344	**-**	0.68 ± 0.09	0.60 ± 0.05
	+	0.45 ± 0.04	0.48 ± 0.03
Δ*hmpA*	**-**	0.66 ± 0.06	0.66 ± 0.15
	+	0.38 ± 0.07	0.49 ± 0.08
Δ*norV*	**-**	0.68 ± 0.06	0.59 ± 0.07
	+	0.22 ± 0.04	0.21 ± 0.05
Δ*nrfA*	**-**	0.72 ± 0.03	0.62 ± 0.02
	+	0.31 ± 0.12	0.34 ± 0.06
Δ*nsrR*	**-**	0.57 ± 0.06	0.57 ± 0.15
	+	0.54 ± 0.09	0.54 ± 0.08
Δ*STM1273*	**-**	0.67 ± 0.04	0.60 ± 0.04
	+	0.47 ± 0.04	0.48 ± 0.04
Δ*ytfE*	**-**	0.70 ± 0.05	0.61 ± 0.04
	+	0.40 ± 0.10	0.46 ± 0.03

As reported in 4.5.1, deletion mutant strains of *hmpA* and *ytfE* do not show any deviations in their sensitivity from the wild-type phenotype (Figure 14). Similarly, deleting *hcr* does not have an increased NO sensitivity under the conditions tested. Hence, these strains show no contribution to nitric oxide detoxification during anaerobic conditions, although it cannot be excluded that functional overlap is masking the loss of these genes. The biggest impact of a single deletion to the growth rate post NO addition is achieved by the deletion of *cstA* or *ygbA.* Less than a third of the unstressed growth rate is observed for each of these strains (Table 18). Neither phenotypes match the hypersensitivity of Δ*norV*, but are in the range of sensitivity shown by the Δ*nrfA* strain.

The Δ*cydB* strain is more resistant to NO than SL1344 and its growth rate during NO exposure is significantly higher than SL1344 two hours after NO addition. This highlights the possible effect of NO on the respiratory chain. The removal of *cydB* might reduce the points of interference of NO with this particular cytochrome oxidase, increasing the amount of other cytochrome oxidases being used, which might be less affected by NO-induced stress.

Deletion strains with significantly different NO sensitivity to the wild-type have been used to create complementation strains, where the gene of interest is re-introduced on an arabinose-inducible plasmid into the deletion strain. All complementation strains display a restored level of sensitivity towards NO that no longer is significantly different to the wild-type (Table 18). This highlights that the phenotypes observed are the result of the specific gene deletion and not originating from a random mutation that might have been caused during *de novo* mutagenesis.

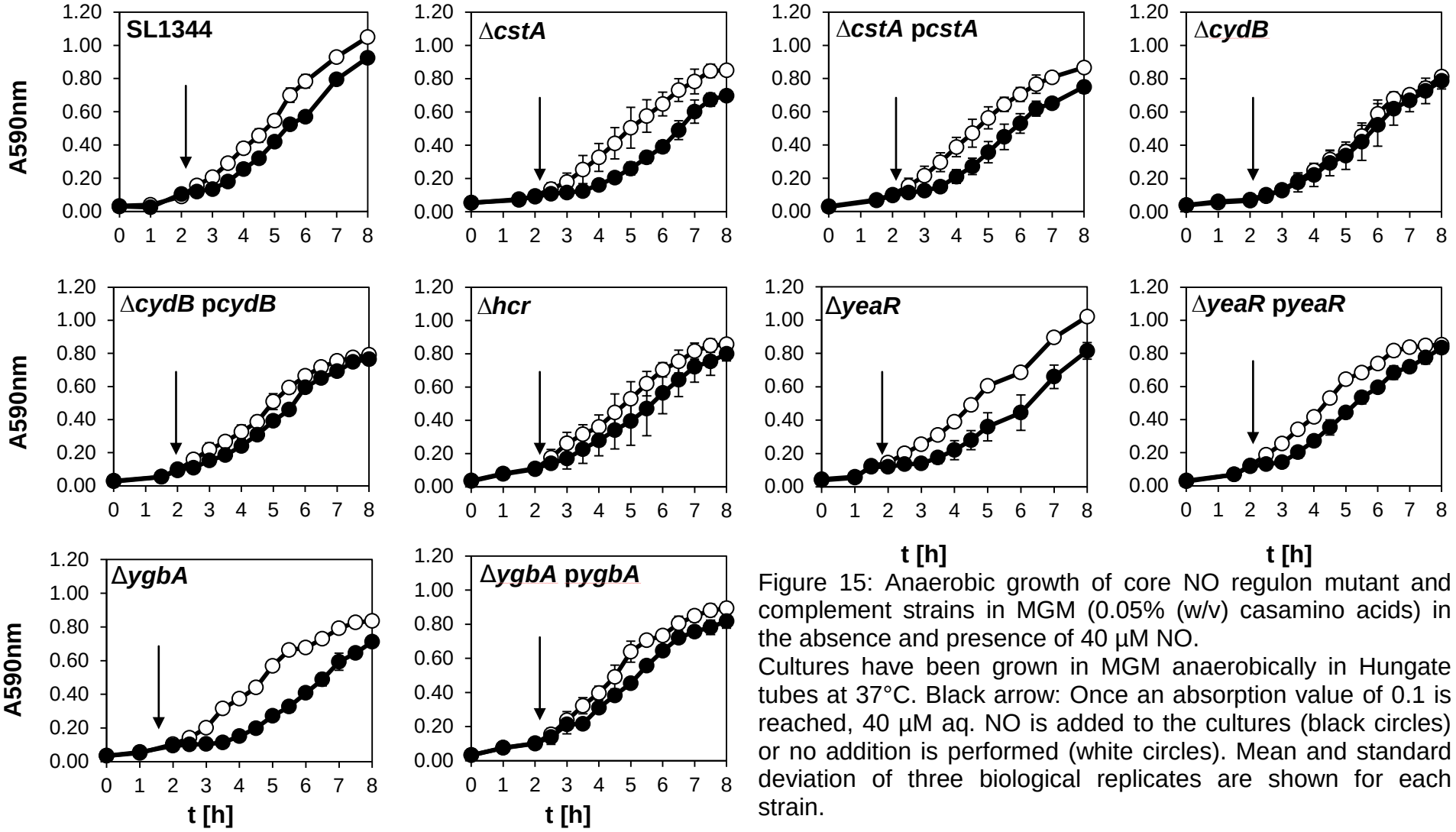


Figure 15: Anaerobic growth of core NO regulon mutant and complement strains in MGM (0.05% (w/v) casamino acids) in the absence and presence of 40 µM NO.
Cultures have been grown in MGM anaerobically in Hungate tubes at 37°C. Black arrow: Once an absorption value of 0.1 is reached, 40 µM aq. NO is added to the cultures (black circles) or no addition is performed (white circles). Mean and standard deviation of three biological replicates are shown for each strain.

Table 18: Growth rates of wild-type and core NO regulon deletion strains from the time of NO addition until two or three hours later, respectively.
The results are the mean of a minimum of three biological and two technical replicates with their standard deviations. Student t tests have been performed and all growth rates with NO are significantly lower in comparison to growth rates without nitric oxide for each strain. Significant differences to corresponding wild-type growth rates are indicated by grey colouration.

Strain	**NO**	**Growth rate ± st. dev.**	
		2 h	**3 h**
SL1344	**-**	0.68 ± 0.09	0.60 ± 0.05
	+	0.45 ± 0.04	0.48 ± 0.03
Δ***cstA***	**-**	0.61 ± 0.04	0.57 ± 0.02
	+	0.20 ± 0.05	0.33 ± 0.03
Δ***cstA*** **p*cstA***	**-**	0.65 ± 0.04	0.57 ± 0.04
	+	0.38 ± 0.10	0.42 ± 0.06
Δ***cydB***	**-**	0.65 ± 0.04	0.55 ± 0.06
	+	0.55 ± 0.06	0.50 ± 0.05
Δ***cydB*** **p*cydB***	**-**	0.63 ± 0.09	0.57 ± 0.05
	+	0.44 ± 0.07	0.46 ± 0.05
Δ***hcr***	**-**	0.62 ± 0.06	0.65 ± 0.17
	+	0.40 ± 0.06	0.42 ± 0.09
Δ***hmpA***	**-**	0.66 ± 0.06	0.66 ± 0.15
	+	0.38 ± 0.07	0.49 ± 0.08
Δ***norV***	**-**	0.68 ± 0.06	0.59 ± 0.07
	+	0.22 ± 0.04	0.21 ± 0.05
Δ***yeaR***	**-**	0.57 ± 0.13	0.53 ± 0.09
	+	0.26 ± 0.11	0.33 ± 0.08
Δ***yeaR*** **p*yeaR***	**-**	0.62 ± 0.08	0.56 ± 0.06
	+	0.43 ± 0.05	0.45 ± 0.05
Δ***ygbA***	**-**	0.64 ± 0.04	0.57 ± 0.03
	+	0.23 ± 0.04	0.35 ± 0.04
Δ***ygbA*** **p*ygbA***	**-**	0.65 ± 0.04	0.59 ± 0.03
	+	0.55 ± 0.06	0.49 ± 0.04
Δ***ytfE***	**-**	0.70 ± 0.05	0.61 ± 0.04
	+	0.40 ± 0.10	0.46 ± 0.03

4.5.3 Sensitivity of deletion strains involved in tellurite resistance

The core NO regulon includes the putative tellurite resistance domain containing gene *yeaR*. In order to examine the influence of such a domain on NO sensitivity, several other genes are also included in the experiment: *STM1808* also contains the DUF1971 and the operon *tehAB* has been annotated for conferring resistance to tellurite for *E. coli* in the past (Avazeri et al, 1997; Taylor et al, 1994; Turner et al, 1995b). In addition, all three genes show significantly increased levels of gene expression after the addition of NO as shown in Table 13. Single mutant strains and a triple deletion strain have been constructed and tested for their sensitivity towards NO under anaerobic conditions. The resulting growth phenotypes can be seen in Figure 16.

As discussed in 4.5.2, a deletion strain lacking *yeaR* shows increased sensitivity of anaerobic growth towards NO addition in this experimental setup. Previously, no aerobic NO sensitivity has been observed (Karlinsey et al, 2012).

A similar level of sensitivity can be seen for strains with deletions of *STM1808*, *tehA* and *tehB*, reducing the growth rate to 0.24, 0.32 and 0.32, respectively. Re-introduction of *yeaR*, *tehB* and *STM1808 in trans* into the respective single deletion strains restores the wild-type sensitivity levels and hence we are able to fully complement the phenotypes of the single deletion strains (Table 19). The deletions of the individual putative tellurite resistance genes lead to a growth rate reduction to ~ 40% of non-exposed cultures.

The sensitivity of the triple deletion strain (Δ*STM1808 tehB yeaR*) shows an accumulation of sensitivities from the three single deletion strains and the delay in growth is extended to three hours post NO addition. The growth rate of Δ*STM1808 tehB yeaR* is at 26% of the rate without NO present (0.17 vs. 0.65), in comparison to a rate of 66% for the wild-type growth (0.45 vs. 0.68). The recovery of this triple deletion strain is very slow and clearly demonstrates for the first time the contribution of these genes to either *Salmonella* NO detoxification or NO-induced damage repair irrespective of their gene annotations of conveying tellurite resistance.

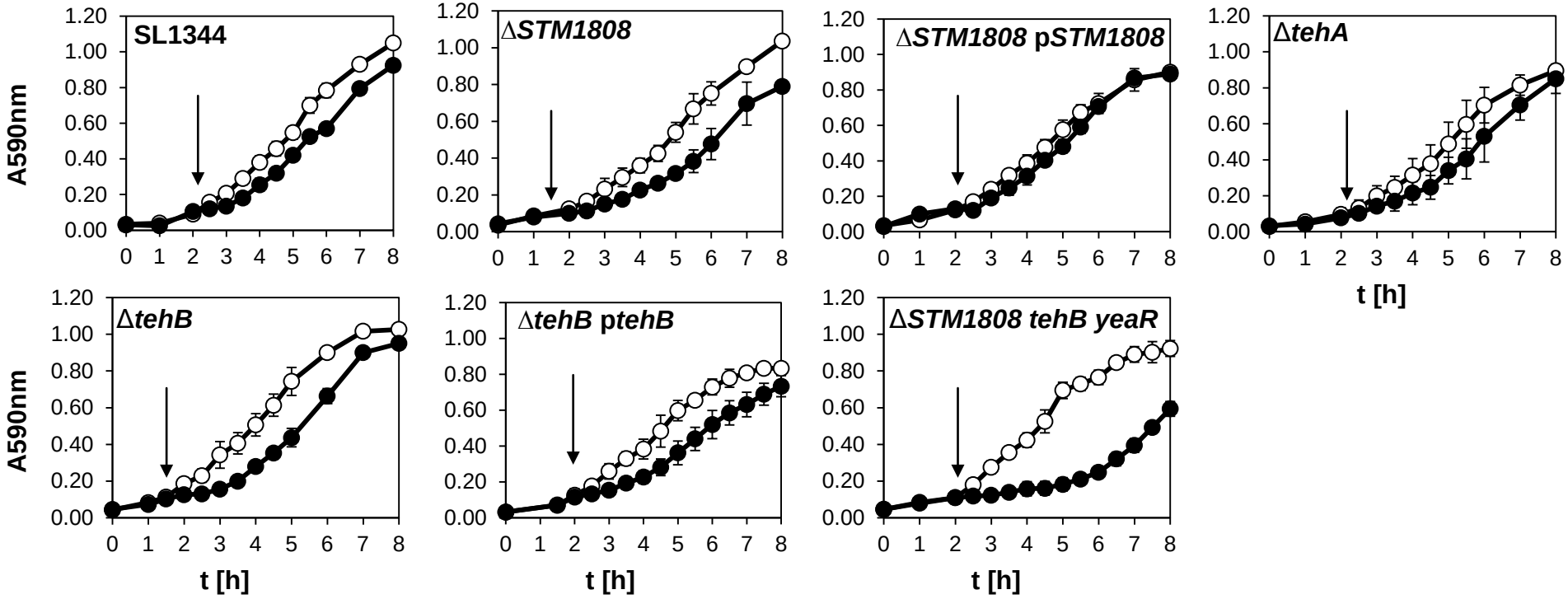


Figure 16: Anaerobic growth of tellurite resistance mutant strains in MGM (0.05% (w/v) casamino acids) in the absence and presence of 40 µM NO.

Cultures have been grown in MGM anaerobically in Hungate tubes at 37°C. Black arrow: Once an absorption value of 0.1 is reached, 40 µM aq. NO is added to the cultures (black circles) or no addition is performed (white circles). Mean and standard deviation of three biological replicates are shown for each strain.

Table 19: Growth rates of wild-type and tellurite resistance deletion strains from the time of NO addition until two or three hours later, respectively.
The results are the mean of a minimum of three biological and two technical replicates with their standard deviations. Student t tests have been performed and all growth rates with NO are significantly lower in comparison to growth rates without nitric oxide for each strain. Significant differences to corresponding wild-type growth rates are indicated by grey colouration.

Strain	**NO**	**Growth rate ± st. dev.**	
		2 h	**3 h**
SL1344	**-**	0.68 ± 0.09	0.60 ± 0.05
	+	0.45 ± 0.04	0.48 ± 0.03
Δ*STM1808*	**-**	0.59 ± 0.05	0.52 ± 0.06
	+	0.24 ± 0.04	0.26 ± 0.05
Δ*STM1808* p*STM1808*	**-**	0.62 ± 0.06	0.56 ± 0.07
	+	0.34 ± 0.11	0.44 ± 0.03
Δ*STM1808 tehB yeaR*	**-**	0.65 ± 0.03	0.58 ± 0.04
	+	0.17 ± 0.11	0.15 ± 0.06
Δ*tehA*	**-**	0.60 ± 0.06	0.55 ± 0.06
	+	0.32 ± 0.05	0.38 ± 0.05
Δ*tehB*	**-**	0.64 ± 0.03	0.57 ± 0.02
	+	0.31 ± 0.09	0.40 ± 0.04
Δ*tehB* p*tehB*	**-**	0.62 ± 0.13	0.57 ± 0.09
	+	0.39 ± 0.04	0.38 ± 0.03
Δ*yeaR*	**-**	0.57 ± 0.13	0.53 ± 0.09
	+	0.26 ± 0.11	0.33 ± 0.08
Δ*yeaR* p*yeaR*	**-**	0.62 ± 0.08	0.56 ± 0.06
	+	0.43 ± 0.05	0.45 ± 0.05

4.6 Discussion

The aim of this chapter has been to determine the contribution of the core NO regulon as well as the remaining NsrR regulated genes to anaerobic detoxification of NO. Experimental confirmation of NsrR regulation of *STM1808* has been another aim.

The results of the β-galactosidase assay have experimentally proven the previously computational identification of an NsrR-binding site upstream of *STM1808* that since has been published by Karlinsey *et al*. in 2012. Therefore, the regulation of *STM1808* expression is explained as depicted in Figure 17. Fully assembled, NsrR contains an iron-sulphur cluster that binds to a specific DNA-binding site once two proteins have dimerized. The dimer prevents the initiation of *STM1808* transcription by blocking the binding site of the RNA polymerase (Figure 17A). In the presence of NO, the [Fe-S] cluster is nitrosylated, where NO replaces the iron. As a result, the DNA binding of NsrR is eliminated and the nitrosylated NsrR dissipates (Figure 17B). This exposes the RNA polymerase binding site of *STM1808* so that RNA polymerase binding and hence transcription of *STM1808* takes place (Figure 17C). Direct binding has been confirmed through the β-galactosidase assay, where the STM1808 promoter has been linked to the *lacZ* gene, encoding for a β-galactosidase. If RNA polymerase binding to the promoter is blocked, no enzyme will be produced and no colour change is observed. This has been observed for the combination of *STM1808* promoter in an NsrR-producing strain. In an NsrR deficient strain, a colour change has indicated that *lacZ* transcription is possible and confirms the expressional regulation of *STM1808* by NsrR.

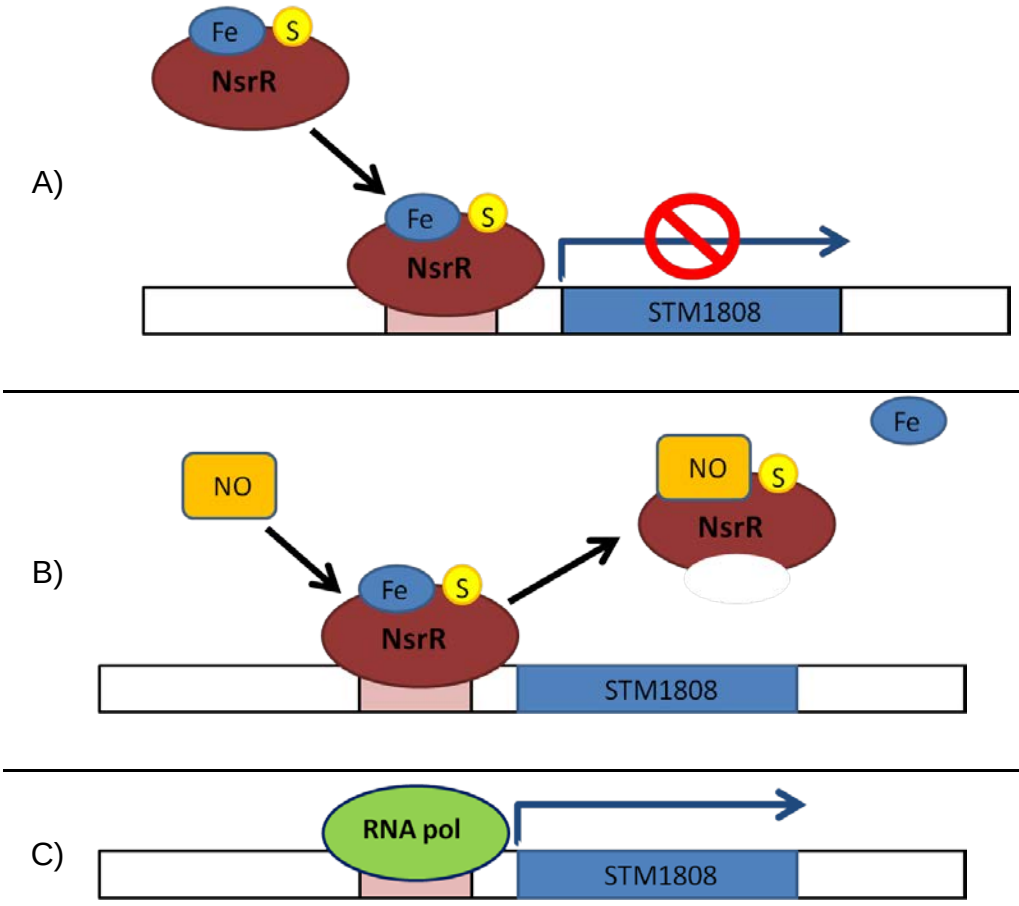


Figure 17: Transcriptional regulation of *STM1808* by NsrR

(A) After protein assembly, NsrR contains a [Fe-S] cluster, binds to its binding site and hence masks the RNA polymerase binding site (pink) in the upstream region of *STM1808*. Therefore no transcription of *STM1808* takes place as the RNA polymerase is unable to bind and to initiate transcription.

(B) The reactivity of NO removes the iron from the [Fe-S] cluster of NsrR, resulting in a conformational change of the repressor. The binding of NsrR to its binding site is no longer possible.

(C) RNA polymerase now binds and initiates transcription of *STM1808*.

No sensitivity is observed when *yeaR* and *STM1808* deletion strains are subjected to 5mM Spermine/NONOate (Sper/NO) in the presence of oxygen (Karlinsey et al, 2012). However, differences in sensitivity due to oxygen concentrations have been reported for Δ*hmpA* in the past (Mills et al, 2008) and it has been recognized that enzyme activities vary due to different oxygen requirements. In addition, the effects of NO have been reported to be most prominent in the absence of oxygen and this is reflected by the physiological conditions found during infection. In the given conditions, the deletion of single tellurite resistance annotated genes or a combination of three results in severely and significantly increased NO sensitivity. Further analysis is necessary to investigate whether these genes *STM1808*, *yeaR* and *tehAB* confer tellurite resistance and whether there could be a link between the two stress-conferring compounds.

The sensitivity levels of the known NO detoxification genes *hmpA*, *norV* and *nrfA* are in agreement with previously published results. Only a minor role for HmpA in anaerobic detoxification of NO is found and the observed wild-type sensitivity towards the addition of NO under anaerobic conditions therefore corresponds with the results of other groups (Crawford & Goldberg, 1998a; Karlinsey et al, 2012; Mills et al, 2008; Stevanin et al, 2002). Similar, the annotated nitric oxide reductase STM1273 has no significant effect on anaerobic NO sensitivity. The biggest contribution to NO defence comes from NorV, where a deletion results in a growth rate reduction to 32%. Similar, high NO sensitivity has been reported for *norV* deletion strains in *E. coli* and *Salmonella* (Baptista et al, 2012; Gardner et al, 2002; Hutchings et al, 2002; Mills et al, 2008). Deleting *nrfA* also leads to an increase in NO sensitivity in previous studies (Mills et al, 2008; Poock et al, 2002). The results shown here therefore support the major contributions of NorV and NrfA as well as the minor contribution of HmpA for NO detoxification that has been previously reported.

Of particular interest in this assay are, however, the core NO regulon genes, the majority regulated by NsrR. The deletion of NsrR results in the de-repression of NsrR regulation that is shown to decrease *Salmonella*'s NO sensitivity significantly. Apart from the NO detoxification genes *hmpA* and *norV*, the core NO regulon also contains the NsrR-regulated genes *hcr*, *yeaR-yoaG*, *ygbA* and

ytfE. The Δ*hcr* strain does not shown any de- or increased NO sensitivity anaerobically and previously shown aerobically increased sensitivity is dependent on the growth medium (Karlinsey et al, 2012). Therefore, sensitivity might be dependent on oxygen and nutrient availability. YtfE has been highlighted for its ability to repair [Fe-S] clusters. This activity is of particular importance during NO exposure due to nitric oxide reactivity with iron, known to interfere with [Fe-S] cluster structure. Hence, deletion of *ytfE* is expected to increase NO sensitivity, unless functional redundancy is able to compensate for the lack of *ytfE*. In line with previous studies in *Salmonella*, Δ*ytfE* is not significantly more NO-sensitive than the parent strain (Gilberthorpe et al, 2007; Karlinsey et al, 2012; Kim et al, 2003). Mice infection studies, however, highlight a role for YtfE during infection as a deletion lowers the oral lethal dose significantly (Kim et al, 2003).

Both *ygbA* and *yeaR* deletion strains display significantly increased sensitivity towards NO: Both strains have a growth rate reduced to 36% and 46% of non-exposed cells, respectively. During aerobic conditions, a previous study has only identify NO sensitivity for Δ*ygbA* in minimal medium, but neither Δ*ygbA* nor Δ*yeaR* are NO sensitive when grown in LB (Karlinsey et al, 2012). Therefore, oxygen and nutrient requirements also play a role for the activity of YeaR and YgbA or their activity might be masked by functional redundancy. The sensitivity levels of Δ*yeaR* and Δ*ygbA* measured in this study compare closely with Δ*norV* and Δ*nrfA*, stressing the importance of YgbA and YeaR during anaerobic NO detoxification.

Although *cstA* regulation is independent of NsrR, the level of sensitivity to NO of a Δ*cstA* strain is equally low to Δ*norV* and Δ*ygbA*. This is the first report of NO sensitivity of a *cstA* deletion strain as this gene so far has not been linked to NO defence, but rather to carbon starvation.

The cytochrome *d* ubiquinol oxidase subunit II encoding gene *cydB* is shown to significantly decrease the NO sensitivity of *S*. Typhimurium. As CydB is part of the respiratory chain, an increase in sensitivity is expected in line with previous findings that Δ*cydA* leads to decreased virulence in mice and that NO inhibits the enzyme activity of CydAB in *E. coli* (Borisov et al, 2004; Stevanin et al, 2000; Turner et al, 2003). Possibly, the loss of *cydB* is compensated for during

in vitro conditions, whereas the more complex *in vivo* environment relies more heavily on CydAB functionality.

Next, the deletion strains lacking putative tellurite resistance genes have been tested for their NO sensitivity. *STM1808* and *yeaR* share the DUF1971 that has been labelled as a tellurite resistance marker. *STM1808* and *tehAB* are also up-regulated in the presence of exogenous NO. The single deletion of either four genes results in significantly increased NO sensitivity that is fully complemented with the introduction of the respective gene on a plasmid. The level of sensitivity ranges from a decrease to 53% (Δ*tehA*) down to 41% (Δ*STM1808*). This is the first demonstration that tellurite resistance genes provide protection against NO. Aerobically, only NO sensitivity for Δ*STM1808* is shown, but this has been dismissed to be related to the putative tellurite resistance function (Karlinsey et al, 2012).

The results presented here clearly show that these genes have an important role for NO defence for *S*. Typhimurium during anaerobic conditions. The deletion of all three tellurite resistance genes *tehB*, *yeaR* and *STM1808* results in a severely growth-impaired strain in the presence of physiological amounts of NO. Irrespective of the gene annotations, it has to be pointed out that these genes provide a significant contribution to *Salmonella*'s NO tolerance. Although all three genes have been annotated to contain tellurite resistance domains, it is still unclear whether *yeaR*, *tehAB* and *STM1808* allow for tellurite resistance in *S*. Typhimurium. It has been reported that their domains for binding *S*-adenosyl-L-methionine (SAM) are truncated and might not fulfil the function of TehB as a SAM-dependent methyltransferase as it has been shown for *Haemophilus influenzae* (Karlinsey et al, 2012). Further analysis is necessary to establish their role for tellurite resistance.

It is concluded that the nutritional environment and oxygen supply play an integral part for the influence of different genes on the sensitivity of *S*. Typhimurium. The role of *norV* and *nrfA* for anaerobic NO detoxification is confirmed. From the core NO regulon, several deletion strains have highlighted the potential involvement of more genes for NO defence. In addition, the tellurite resistance genes have pointed out another group of genes that might have evolved to support *Salmonella*'s survival during infection. Their potential for tellurite detoxification also needs to be investigated further.

5 Contribution of NO detoxification systems to tellurite resistance

5.1 Introduction to tellurium, tellurite and tellurite toxicity

5.1.1 Tellurium

Tellurium (Te) belongs to the group of chalcogenide elements together with oxygen, sulphur, selenium and polonium. Unlike other group members, tellurium is not an essential biological element (Ba et al, 2010). Tellurium concentrations naturally vary across the world and higher concentrations have been measured in the presence of copper and sulphur ores (Chasteen et al, 2009). In industry, a range of applications are found for this element (Ba et al, 2010; Turner et al, 2012). Tellurium has been used in metallurgy to improve the properties of steel and as a component in solar panels. The spectroscopic properties of tellurium have led to its use in fluorescent Cadmium/Tellurium quantum dots that are used as measuring probes, e.g. to determine tiopronin levels in clinical samples (Wang et al, 2008). In addition, tellurium is increasingly found in rechargeable batteries. There are three groups of tellurium compounds: (i) complex tellurium-containing structures, (ii) organotellurides and (iii) inorganic tellurides (reviewed by Ba *et al.*, 2010). Tellurium-containing structures have a central Te atom and have a range of different ligands. Organotellurides have di- or trivalent Te atoms and alkyl or aryl residues. Organotellurides are chemically unstable and are redox active as they are oxidized by ROS or reduced by thiols. Inorganic tellurides form acids such as tellurious acid (H_2TeO_3) or telluric acid (H_2TeO_4). The salts of tellurious and telluric acid, tellurite (TeO_3^{2-}) and tellurate (TeO_4^{2-}) respectively, are well soluble and are toxic to bacteria and mammals (Chasteen et al, 2009). The reduction of tellurite and tellurate to Te^0 or the methylation to yield $(CH_3)_2Te$ make use of metabolic selenium pathways within the cell.

5.1.2 Tellurite

Historically, tellurite (TeO_3^{2-}) has been used in the 1930s as an antibiotic to treat infections such as tuberculosis and dermatitis (Molina-Quiroz et al, 2012; Taylor, 1999). Tellurium toxicity depends on its oxidation state as well as whether it is present in organic or inorganic form. Ingestion or inhalation of tellurium compounds leads to nausea, vomiting and garlic odour (Ba et al, 2010; Prigent-Combaret et al, 2012). In general, the toxicity of tellurite stems from its reactivity with several components of proteins such as cysteines, selenium and

sulphur (Ba et al, 2010; Taylor, 1999). Free Fe(II) within the cell consequently causes the release of ROS as it fuels the Fenton reaction and further ROS are generated as a result of tellurite reduction directly (Chasteen et al, 2009). The addition of tellurite to growth media allows the selection of bacteria such as *Shigella* spp. and *S. aureus* as they form grey to black colonies on tellurite-containing agar (Chasteen et al, 2009; Taylor, 1999). A list of properties of tellurite can be found in Table 20.

5.1.3 Mode of action of tellurite

Tellurite interacts with cysteine residues on proteins, binds to selenium within proteins and replaces sulphur groups (Ba et al, 2010; Taylor, 1999). Defects in cysteine metabolism increased the sensitivity *E. coli* to tellurite (Chasteen et al, 2009; Fuentes et al, 2007). The importance of cysteine to bacterial tellurite resistance lies in cysteine's protection against oxidative damage. Cysteine is an essential component required for the biosynthesis of glutathione (GSH) that serves as a reducing agent of tellurite as well as a scavenger of ROS. The *in vitro* reduction of tellurite has been shown to release hydroxyl radicals as part of the Fenton reaction and is described in the following reaction: $TeO_3^{2-} + 3\ H_2O + 4\ e^- \rightarrow Te^0 + 6\ OH^-$ (Turner et al, 1999). The increased concentration of hydroxyl radicals results in oxidative stress, oxidizes thiols and induces the activity of the superoxide dismutase to counteract ROS toxicity (Borsetti et al, 2005; Pérez et al, 2007; Turner et al, 2012). Any defects in either the glutathione or thioredoxin thiol systems impair *E. coli*'s natural resistance to tellurite (Turner et al, 2001; Turner et al, 1999). The reduction of tellurite is also performed by the nitrate reductases NarZ and NarG purified from *E. coli* under aerobic conditions (Avazeri et al, 1997). The same study shows activity of an unknown, anaerobic tellurite reductase, but no further characterisation has been performed. Tellurite reductase activity of Nap has not been examined in the Avazeri study.

Table 20: Summary of properties of tellurite

Chemical properties:

- inorganic salt of tellurium
- soluble
- toxic
- biocidal

Uses:

- alloy for electrical, optical and thermal equipment
- bacterial selection
- historically as an antibiotic
- potential anti-cancer drug

Toxicity:

- reactivity with cysteines
- replaces sulphur
- oxidizing
- binds to selenium
- release of ROS upon reduction

Detoxification/conversion

- reduction to tellurium, releasing OH^- → Te^0 deposits
- methylation → excretion

Resistance:

- on plasmids:
  - *klaAB telB*
  - *ars* operon
  - *ter* operon
- genomic:
  - *tehAB*
  - *tmp*
  - *trgAB cysK*

Methylation of tellurite and efflux of the metal have been proposed as additional mechanisms to provide protection against tellurite toxicity. Methylation of tellurite by the bacterial thiopurine methyltransferase has been demonstrated and the characteristic garlic smell created by dimethyl telluride is observed (Prigent-Combaret et al, 2012). Although the efflux of tellurite outside of the cell has been proposed as a general resistance mechanism, it has not yet been shown for *E. coli* (Chasteen et al, 2009; Turner et al, 1995a). Further protection against tellurite toxicity has been shown to originate from reductase activity of the NAD(P)H-dependent catalases of *Staphylococcus epidermidis* and *E. coli* (Calderon et al, 2006).

Tellurite inactivates [4Fe-4S] clusters by removing iron, resulting in an inactive $[3Fe\text{-}4S]^{+}$ cluster (Calderon et al, 2009). This not only impacts the protein structure and most likely its activity, but also feeds iron into the Fenton reaction. The liberated Fe(II) generates additional ROS via the Fenton reaction and therefore increases the cellular damage. Deleting enzymes that allow the restoration of [Fe-S] clusters hence increases the tellurite sensitivity. Such enzymes include the cysteine synthase CysK and the cysteine desulfurase IscS (Fuentes et al, 2007; Rojas & Vásquez, 2005; Tantalean et al, 2003).

5.1.4 Tellurite resistance

Although nitrate reductases, catalases and other enzymes with tellurite reduction properties have been identified, they are only able to confer a low level of natural tellurite resistance. In *E. coli* for example, the minimal inhibitory concentration (MIC) lies between 1-2 µg x mL^{-1}, equivalent to 3.94–7.88 µM (Summers & Jacoby, 1977; Taylor et al, 1994). In comparison with other metals such as Zinc (MIC = 1 mM) and Cadmium (MIC = 0.5 mM), the tolerance against tellurite is between 100 and 200 times lower (Chasteen et al, 2009).

In addition to the nitrate reductases and catalases, six tellurite resistance mechanisms have been identified that increase the natural tellurite resistance of *E. coli* from 1µg x mL^{-1} to 128 µg x mL^{-1} (Summers & Jacoby, 1977; Taylor et al, 1994). Three of these mechanisms are located on plasmids and have been found in a range of bacteria, but their precise mechanisms remain unknown (Chasteen et al, 2009; Walter & Taylor, 1989) (see Table 21).

Table 21: Tellurite resistance determinants.

Their origins as determined by DNA sequencing and example organisms containing homologous genes are shown, adapted from Taylor, 1999.

Origin:	**Gene(s):**	**Homologues in other organisms:**
E. coli K-12	*tehA*	*Arabidopsis thaliana* *Archaeoglobus fulgidus* *H. influenzae* *Methanocaldococcus jannaschii* *Pyrococcus horikoshii*
	tehB	*Aggregatibacter acetinomycetemcomitans* *Eikenella corrodens* *H. influenzae* *N. gonorrhoea* *N. meningitidis*
IncP (RP4)	*klaA (kilA)*	
	klaB (telA)	*B. subtilis* *Deinococcus radiodurans* *Rhodobacter sphaeroides* *Y. pestis*
	klaC (telB)	*Agrobacterium tumefaciens* *Enterobacter aerogenes* *Rhizobium sp.*
R773	*ars* operon	
IncHI2 (R478)	*terZ*	*B. subtilis* *Clostridium acetobutylicum* *D. radiodurans* *Dictyostelium discoideum* *P. aeruginosa*
	terA	*C. jejuni* *C. acetobutyllicum* *D. radiodurans* *Y. pestis*
	terB	*D. radiodurans* *Y. pestis*
	terC	*E. coli* K-12 *M. tuberculosis* *Myxococcus xanthus* *N. menigitidis* *Pseudomonas aeruginosa* *Rickettsia prowazekii* *Y. pestis*
	terD	*B. subtilis* *D. radiodurans* *D. discoideum* *Y. pestis*
	terE	*B. subtilis* *D. radiodurans* *D. discoideum*

Origin:	Gene(s):	Homologues in other organisms:
IncH12 (cont.)	*terE (cont.)*	*Y. pestis*
	terF	*B. subtilis* *D. radiodurans*
	terF	*Y. pestis*
P. syringae	*tmp*	*Homo sapiens* *Mus musculus* *M. tuberculosis* *P. aeruginosa*
R. sphaeroides	*trgA*	
	trgB	
	cysK	*A. thaliana* *B. subtilis* *Brassica juncea* *D. discoideum* *Emericella nidulans* *E. coli* K-12 *M. tuberculosis* *Schizosaccharomyces pombe* *Sunechocystis sp.*

Ter operon

The plasmid-located *ter* operon confers resistance to colicin, a pore-forming bacterial toxin, in *E. coli* as well as bacteriophage infection and tellurite resistance (Kormutakova et al, 2000; Whelan et al, 1997). Another plasmid encoded operon is *arsABC*, encoding for an anion-translocation ATPase (Turner et al, 1992). ArsABC usually maintains a low intracellular concentration of arsenite and arsenate within the bacterial cell, but has also been shown to decrease the intracellular concentration of tellurite by 55%. Expression of *arsABC* raises the MIC of *E. coli* towards tellurite to 64 µg x mL^{-1}. The plasmid-located genes *kilA*, *telA* and *telB* have also been shown to confer tellurite resistance, however, again, their mode of action is not clear (Taylor, 1999). When *kilA* is over-expressed in *E. coli*, it increases tellurite resistance independent of the growth conditions (Turner et al, 1995b).

TrgAB

The *trgAB* genes of *R. sphaeroides* are associated with the bacterial membrane and contribute to tellurite resistance (Taylor, 1999). Also, the *R. sphaeroides* and *E. coli* cysteine synthases CysK have been linked with tellurite resistance, presumably because it can counterbalance dysfunctional cysteines that have reacted with tellurite by ensuring the synthesis of new cysteines (Fuentes et al, 2007; Taylor, 1999).

TehAB

The chromosomally located operon *tehAB* has first been thought to be located on a plasmid, but has later been identified within the *E. coli* genome (Taylor et al, 1994). An increase of *tehAB* in copy number when the operon is cloned into a multi-copy plasmid increases the MIC of *E. coli* to levels between 128 and 256 µg x mL^{-1} (Avazeri et al, 1997; Taylor et al, 1994; Turner et al, 1995b). An efflux property has been proposed for TehA, but although it has been shown to provide flux activity for proflavin and ethidium bromide, no efflux activity for tellurite has been identified (Taylor, 1999; Turner et al, 1997).

The *tehB* gene is not unique to *E. coli*, but homologues have been found in a range of bacteria (Table 21). Deletion of *tehB* in *H. influenzae* does not only increase tellurite sensitivity, but also hydrogen peroxide sensitivity as well as impair the infection ability in a rat infection model. The effect of a *tehB* deletion in *H. influenzae* has been complemented with *E. coli tehB* (Whitby et al, 2010).

Structural analysis of purified TehB protein has highlighted that TehB does not specifically bind to tellurite, but also binds selenite and selenate, the toxic anions of selenium (Choudhury et al, 2011). Previously, TehB has been identified as an *S*-adenosyl-L-methionine (SAM)-dependent methyltransferase that could convert tellurite into dimethyl telluride by a similar pathway employed for selenate and selenite methylation (Liu et al, 2000). Dimerisation and a conformational change upon binding of SAM and tellurite have also been identified (Dyllick-Brenzinger et al, 2000). The regulation of this operon has been investigated in the past. In *E. coli*, initially no NsrR binding site has been found during computational analysis of the genome, but weak NsrR regulation and binding sites have been shown experimentally (Bodenmiller & Spiro, 2006;

Partridge et al, 2009; Rodionov et al, 2005). The *S*. Typhimurium genome contains NsrR binding sites upstream of *tehAB*, but experimental evidence highlights that the regulation by NsrR is weak (Gilberthorpe et al, 2007; Karlinsey et al, 2012; Rodionov et al, 2005). Exposure to NO increases the expression of *tehAB* as does the nitrate-rich environment encountered during urinary tract infections e. g. of humans, indicating that possibly other regulators are also involved and might be masking NsrR regulation (Justino et al, 2005; Roos & Klemm, 2006).

Homologues of the *H*. *influenzae* TehB protein have been found in *Salmonella* and *S*. Typhimurium has three TehB-like proteins: STM1808, TehB and YeaR (Karlinsey et al, 2012). STM1808 and YeaR contain the domain of unknown function DUF1971 and share over 70% protein similarity (Figure 18). Both contain the conserved histidines H32 and H82 of the DUF that for STM1808 have been shown to be required for NO resistance, whereas histidines at other positions within the protein (H31, H95 and H 102) are not required (Karlinsey et al, 2012). Further characterisation indicated that H32 and H82 allow for the coordination and binding of a Zinc ion. Initial homology studies with TehB of *H. influenzae* have highlighted truncation of *S*. Typhimurium and *E. coli* TehB proteins at the C-terminal domain. Therefore, it has been concluded that STM1808 and YeaR would not possess SAM-dependent methyltransferase activity and hence would not be able to reduce tellurite. No tellurite sensitivity assays with the *STM1808* and *yeaR* deletion strains have been performed to further validate these conclusions.

```
STM1808 MSHLRIPANWKVKRSTPFFTKENVPAALLSHHN---TAAGVFGQLCVMEGTVTYYGFANE
  yeaR --MRQIPQNHIHTRSTPFWNKETAPAGIFERHLDKGTRPDVYPRLSVMQGAVKYLGYADE

STM1808 TATEPEVKVVINAGQFATSPPQYWHRVELS-DDARFNIHFWVEEDHQGEEMYQQKKA---
  yeaR HCSEPEEIMVINAGEFGVFPPEKWHNIEVMTDDTYFNIDFFVAPEVLMEGATSRKVIHAG
```

Figure 18: Alignment of the amino acid sequences of *S*. Typhimurium genes *STM1808* and *yeaR*.

The DUF1971 sequence is shown in bold and the conserved histidines H32 and H82 are shaded in grey. Over 70% of amino acids are shared between both sequences.

As shown in previous chapters, *yeaR* has been shown to respond to exposure to endogenous and exogenous NO sources. Additionally, deletion of *yeaR* results in increased NO sensitivity, raising the question whether there could be a link between NO and tellurite sensitivity and/or whether the genomic annotations of *yeaR* and *STM1808* containing putative tellurite resistance domains are misleading.

5.2 Aim

The aim of this chapter is to determine whether the putative tellurite resistance and NsrR regulated genes contribute to tellurite resistance. This study aims to identify whether there is a correlation between tellurite and NO resistance. Further, the changes in gene expression levels of tellurite resistance and NO detoxification genes are investigated.

5.3 Methods

All strains are grown aerobically at 37°C as described in 2.1.3. Overnight cultures are grown without tellurite and 0.125 µg x mL^{-1} tellurite is added freshly to the LB medium before the start of the growth curve. Optical density of the cultures is monitored every hour. L-arabinose, DTT and tellurite are added to a subset of the cultures. In order to assess gene expression changes after tellurite exposure, 0.125 µg x mL^{-1} potassium tellurite is added to SL1344 cultures growing in LB in a separate experiment. Cell samples for RNA extraction (described in 2.11) are taken shortly before and 15, 30 and 60 minutes post tellurite addition. Levels of expression are analysed using RT-PCR as described in 2.12 and compared to levels of *ampD* and to levels prior to tellurite addition.

5.4 Results

5.4.1 Growth of strains with deletions of putative tellurite resistance genes in the presence of potassium tellurite

The addition of even low concentrations of 0.125 µg x mL^{-1} tellurite, a tenth of the MIC of *E. coli*, into the growth medium result in severe growth delays in the wild-type strain (Figure 19). In order to assess the importance of putative tellurite resistance genes, single deletion and a triple mutation strain of the tellurite resistance genes *STM1808*, *tehAB* and *yeaR* are tested under the same conditions.

Aerobic LB cultures of the deletion strains without tellurite addition do not display any growth defects. The deletion of *STM1808*, a hypothetical gene with a DUF for tellurite resistance, affects the aerobic growth in LB in the presence of tellurite as growth is significantly lower than the parent strain with a rate of 0.33 in comparison to 0.46 (Table 22). The introduction of *STM1808* on a pBAD plasmid is able to restore the phenotype of the wild-type where only minor growth delays are observed, reflected by the rise in the growth rate to 0.44. The *tehA* deletion strain shows the same phenotype as Δ*STM1808*, with an impaired growth rate of 0.34 in the presence of tellurite. This indicates that both genes significantly contribute to the detoxification of tellurite. The single deletions of *yeaR* and *tehB* respectively, decrease the strains' growth further than deletion of *STM1808* or *tehA* to 0.17 and 0.13, respectively. The re-introduction of the respective genes is able to restore the growth significantly; in the case of Δ*tehB* to wild-type levels of 0.46.

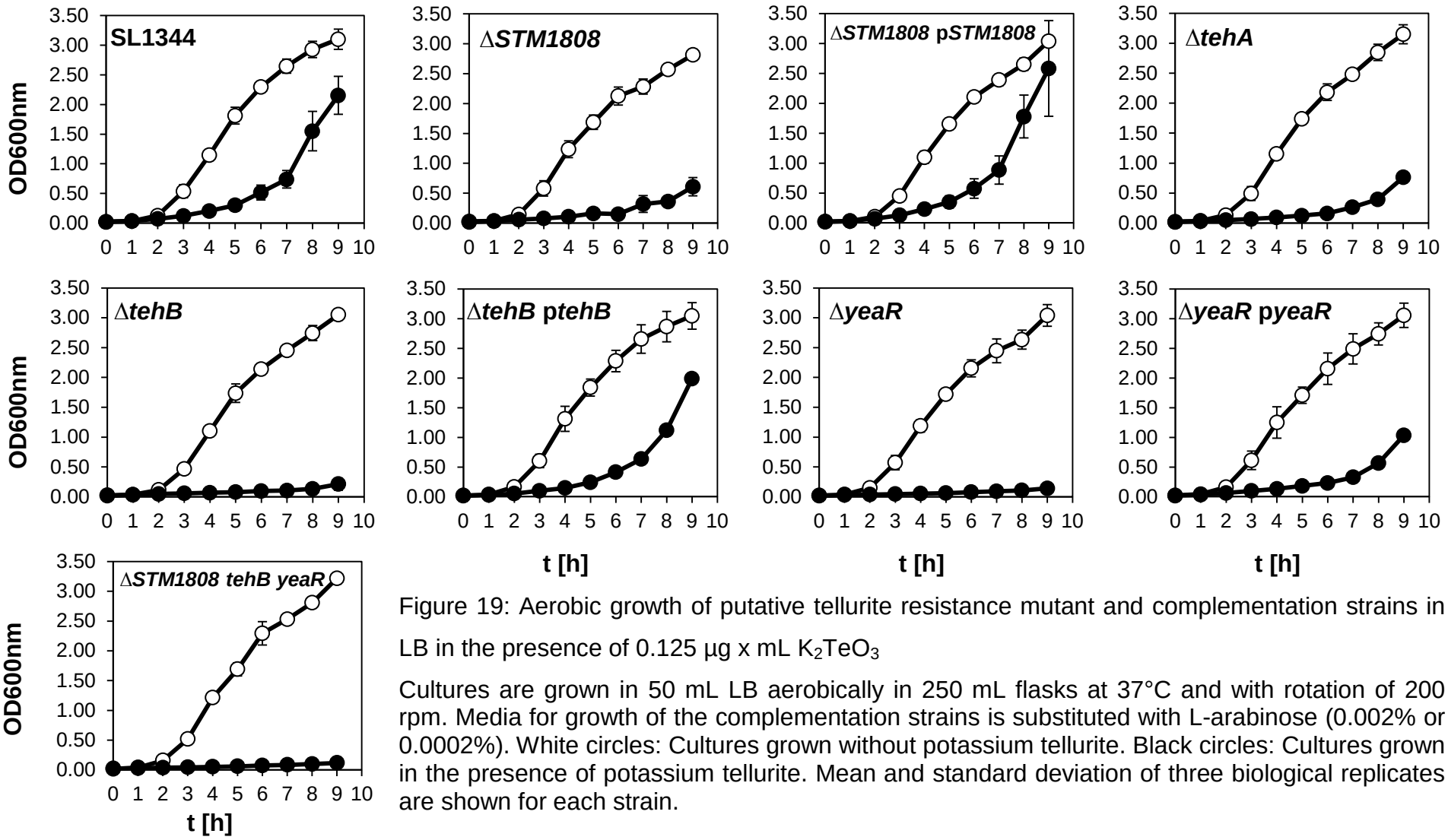


Figure 19: Aerobic growth of putative tellurite resistance mutant and complementation strains in LB in the presence of 0.125 µg x mL K_2TeO_3

Cultures are grown in 50 mL LB aerobically in 250 mL flasks at 37°C and with rotation of 200 rpm. Media for growth of the complementation strains is substituted with L-arabinose (0.002% or 0.0002%). White circles: Cultures grown without potassium tellurite. Black circles: Cultures grown in the presence of potassium tellurite. Mean and standard deviation of three biological replicates are shown for each strain.

Table 22: Growth rates of strains grown in the absence (-) and presence (+) of 0.125 µg x mL^{-1} K_2TeO_3 aerobically in LB with shaking at 200rpm at 37°C.
Media of complementation strains is substituted with L-arabinose. Growth rates have been calculated using the data of a minimum of three biological replicates and evaluating the growth between 3 and 7 hours into the experiment. No significant differences for growth rates in the absence of potassium tellurite in comparison to wild-type have been detected. Grey shading indicates a significant difference between growth rates in comparison to the respective wild-type rate. [1]: Improvement of the deletion mutant phenotype despite significant difference to wild-type phenotype.

Strain:	K_2TeO_3:	Growth rate:
SL1344	-	0.40 ± 0.05
	+	0.46 ± 0.01
Δ*STM1808*	-	0.35 ± 0.05
	+	0.33 ± 0.05
Δ*STM1808* p*STM1808*	-	0.42 ± 0.03
	+[1]	0.44 ± 0.07
Δ*STM1808 tehB yeaR*	-	0.40 ± 0.02
	+	0.14 ± 0.02
Δ*tehA*	-	0.41 ± 0.05
	+	0.34 ± 0.03
Δ*tehB*	-	0.41 ± 0.02
	+	0.13 ± 0.04
Δ*tehB* p*tehB*	-	0.37 ± 0.04
	+	0.46 ± 0.02
Δ*yeaR*	-	0.37 ± 0.05
	+	0.17 ± 0.03
Δ*yeaR* p*yeaR*	-	0.36 ± 0.04
	+[1]	0.30 ± 0.02

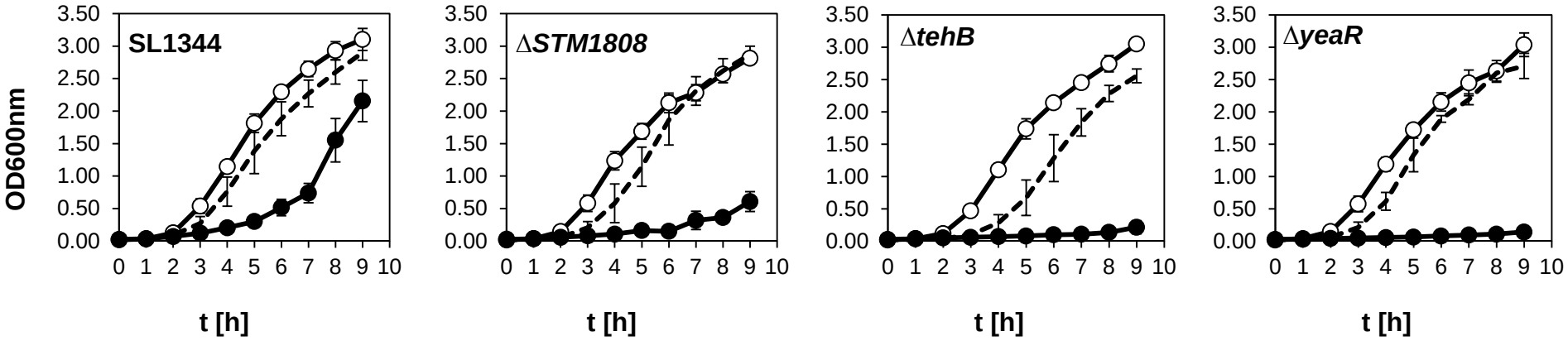


Figure 20: Aerobic growth of putative tellurite resistance mutant strains in LB in the presence of 0.125 µg x mL K_2TeO_3 and 100 µM DTT.

Cultures are grown in 50 mL LB aerobically in 250 mL flasks at 37°C and with rotation of 200 rpm. White circles: Cultures grown without potassium tellurite. Black circles: Cultures grown in the presence of potassium tellurite. Dashed line: Growth of respective strain in LB + 0.125 µg x mL K_2TeO_3 and 100 µM DTT. Mean and standard deviation of three biological replicates are shown for each strain.

5 Contribution of NO detoxification systems to tellurite resistance

The triple deletion of *STM1808*, *tehB* and *yeaR* results in growth as slow as observed for the Δ*tehB* and the Δ*yeaR* strain.

In order to investigate whether the toxicity of tellurite is due to an increase in ROS, the ROS scavenger DTT has been added to a third set of cultures also containing tellurite. The addition of 100 µM DTT is sufficient to allow for the restoration of growth in the wild-type strain (Figure 20). This is also reflected in the growth rate that significantly improved to 0.55 (Table 23). Similar, the growth of the deletion strains is restored.

Table 23: Growth rates of strains grown in the absence (-) and presence (+) of 0.125 µg x mL^{-1} K_2TeO_3 aerobically in LB with shaking at 200rpm at 37°C.
Media of complementation strains is substituted with L-arabinose. Growth rates have been calculated using the data of a minimum of three biological replicates and evaluating the growth between 3 and 7 hours into the experiment. No significant differences for growth rates in the absence of potassium tellurite in comparison to wild-type have been detected. Grey shading indicates a significant difference between growth rates in comparison to the respective wild-type rate. [1]: Significantly increased growth rate in comparison to wild-type equivalent rate.

Strain:	K_2TeO_3:	Growth rate:
SL1344	**-**	0.40 ± 0.05
	+	0.46 ± 0.01
	+K_2TeO_3+ DTT[1]	0.55 ± 0.09
Δ*STM1808*	**-**	0.35 ± 0.05
	+	0.33 ± 0.05
	+K_2TeO_3+ DTT	0.63 ± 0.12
Δ*tehB*	**-**	0.41 ± 0.02
	+	0.13 ± 0.04
	+K_2TeO_3+ DTT	0.73 ± 0.07
Δ*yeaR*	**-**	0.37 ± 0.05
	+	0.17 ± 0.03
	+K_2TeO_3+ DTT	0.60 ± 0.12

5.4.2 Growth of strains with deletions of nitrate and nitrite reduction genes in the presence of potassium tellurite

Previous studies have shown that nitrate reductases reduce tellurite under aerobic conditions. This provides a possible link between NO and tellurite resistance. We therefore have determined the tellurite resistance of strains lacking nitrate and nitrite reductases.

The single deletions of *napDA*, *narG* and *narZ* do not cause any significant growth deviations from the parent strain in the presence of tellurite (Figure 21). The growth rates of the single deletion strains range between 0.42 and 0.55 in comparison to a value of 0.46 of the parent strain (Table 24). However, the growth rate of a triple mutant lacking nitrate reductase operons (Δ*napDA narGHIJ narZYWV*) is significantly impaired in the presence of tellurite and drops to 0.35. In comparison to the effect of a *tehB* deletion (growth rate: 0.13), this mutant strain has only a minor growth delay. The deletion of the cytochrome *c* reductase gene *nrfA* produces a growth delay of similar magnitude to the triple nitrate reductase mutant strain with a value of 0.34. Deleting the nitrite reductase gene *nirB* results in tellurite growth rate of 0.20, ranging just above 50% of the same strain grown in the absence of tellurite. The biggest effect of a single mutation therefore can be attributed to *nirB*, followed by *nrfA*. The ability of nitrate reductases to provide protection against tellurite toxicity is only minor, especially in comparison to the contribution of *nirB* and *nrfA*.

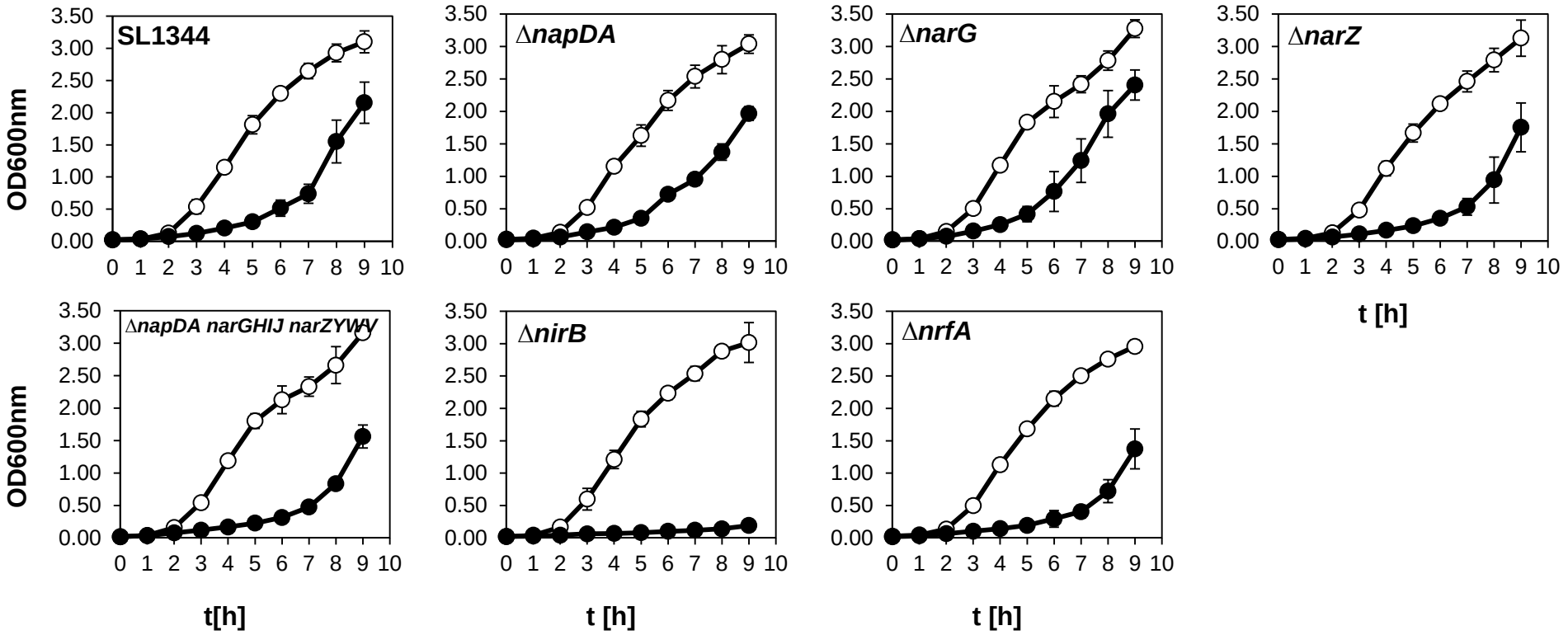


Figure 21: Aerobic growth of nitrate and nitrite reduction mutant strains in LB in the presence of 0.125 µg x mL K_2TeO_3

Cultures are grown in 50 mL LB aerobically in 250 mL flasks at 37°C and with rotation of 200 rpm. White circles: Cultures grown without potassium tellurite. Black circles: Cultures grown in the presence of potassium tellurite. Mean and standard deviation of three biological replicates are shown for each strain.

Table 24: Growth rates of nitrate and nitrite reduction mutant strains grown in the absence (-) and presence (+) of 0.125 µg x mL K_2TeO_3 aerobically in LB with shaking at 200 rpm at 37°C.

Growth rates have been calculated using the data of a minimum of three biological replicates and evaluating the growth between 3 and 7 hours into the experiment. No significant differences for growth rates in the absence of potassium tellurite in comparison to wild-type have been detected. Grey shading indicates a significant difference between growth rates in comparison to the respective wild-type rate.

Strain:	K_2TeO_3:	Growth rate:
SL1344	-	0.40 ± 0.05
	+	0.46 ± 0.01
Δ***napDA***	-	0.42 ± 0.02
	+	0.47 ± 0.01
Δ***napDA narGHIJ narZYWV***	-	0.37 ± 0.05
	+	0.35 ± 0.03
Δ***narG***	-	0.40 ± 0.02
	+	0.55 ± 0.06
Δ***narZ***	-	0.41 ± 0.03
	+	0.42 ± 0.01
Δ***nirB***	-	0.37 ± 0.07
	+	0.20 ± 0.01
Δ***nrfA***	-	0.41 ± 0.01
	+	0.34 ± 0.05

5.4.3 Growth of strains with deletions of known NO detoxification genes in the presence of potassium tellurite

Since there has been an overlap of putative tellurite genes and NO detoxification genes in the core NO regulon, a range of strains with deletions of known NO detoxification genes have also been tested for their tellurite sensitivity. Despite different oxygen requirements for NO detoxification by HmpA, NorV and NrfA, all respective deletion strains are significantly slower growing in the presence of tellurite than the wild-type strain (Figure 22). For Δ*hmpA*, the growth rate is only 50% of the comparable rate without tellurite present and the rate of Δ*norV* and Δ*nrfA* is around 80% (Table 25). The recovery of Δ*hmpA* is achieved when a copy of *hmpA* is re-introduced on a plasmid.

The deletion of the putative nitric oxide reductase gene *STM1273* also results in a significantly slower growing strain in the presence of tellurite, but growth is only decreased by 10% in comparison to the growth absent of tellurite. Complementation of the deletion recovers the growth of Δ*STM1273*. A decrease by 60% in the growth rate is observed for the deletion of the [Fe-S] cluster repair gene *ytfE*. This phenotype is reverted by the introduction of a complementation plasmid.

A significant increase in the growth rate is observed for the *nsrR* deletion strain where the mutant strain grows better in the presence of tellurite than its parent strain. As a known regulator of NO detoxification genes, NsrR de-regulation might benefit the strain's growth in the presence of tellurite if tellurite and its toxic effects are reverted by NO detoxification enzymes.

All in all, the deletion of NO detoxification genes also affects the sensitivity towards tellurite and de-regulation through the deletion of NsrR could enhance the ability to detoxify tellurite.

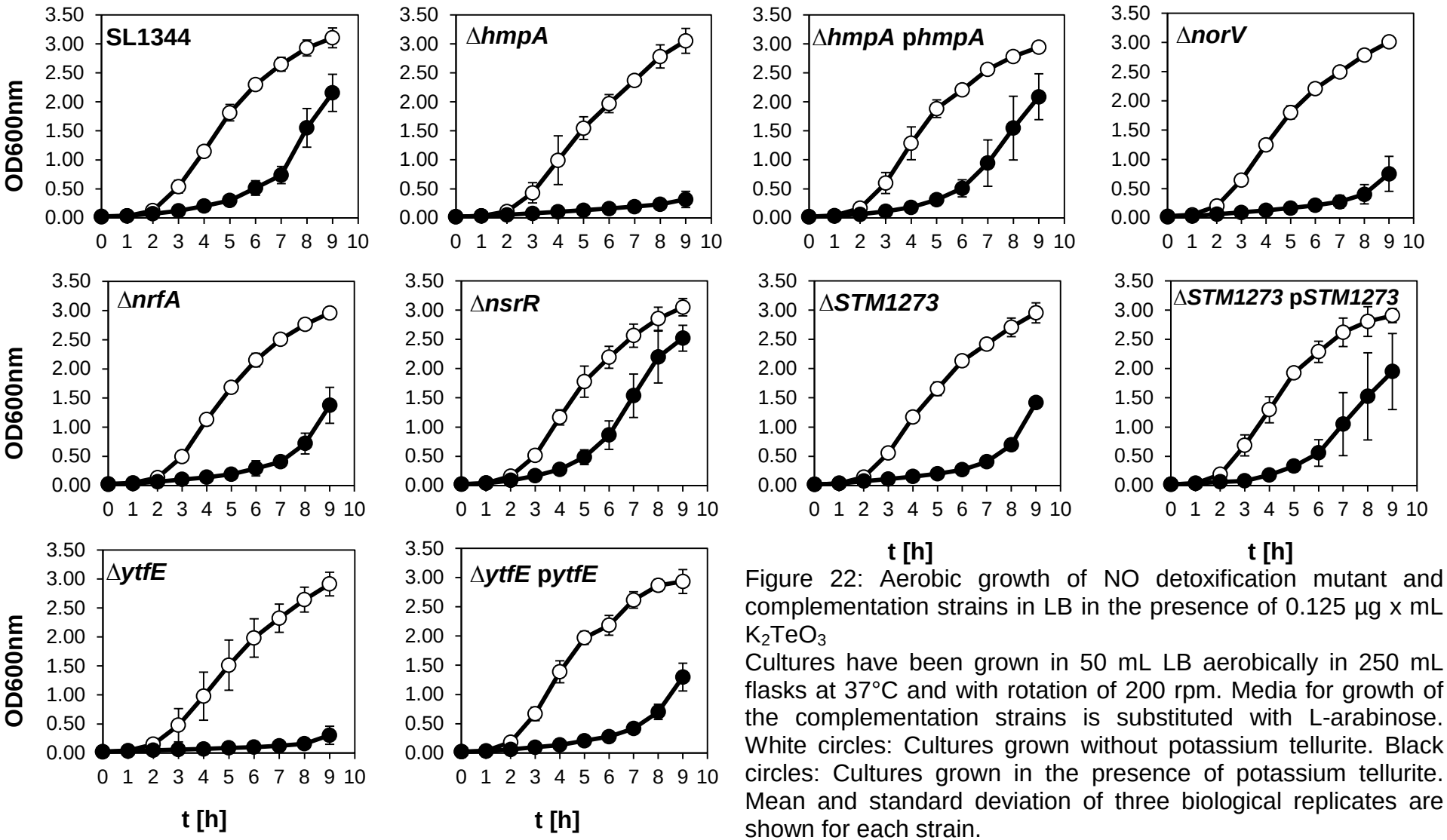


Figure 22: Aerobic growth of NO detoxification mutant and complementation strains in LB in the presence of 0.125 µg x mL K_2TeO_3

Cultures have been grown in 50 mL LB aerobically in 250 mL flasks at 37°C and with rotation of 200 rpm. Media for growth of the complementation strains is substituted with L-arabinose. White circles: Cultures grown without potassium tellurite. Black circles: Cultures grown in the presence of potassium tellurite. Mean and standard deviation of three biological replicates are shown for each strain.

Table 25: Growth rates of strains grown in the absence (-) and presence (+) of 0.125 µg x mL^{-1} K_2TeO_3 aerobically in LB with shaking at 200 rpm at 37°C. Growth rates have been calculated using the data of a minimum of three biological replicates and evaluating the growth between 3 and 7 hours into the experiment. No significant differences for growth rates in the absence of potassium tellurite in comparison to wild-type have been detected. Grey shading indicates a significant difference between growth rates in comparison to the respective wild-type rate. [1]: Significantly increased growth rate in comparison to wild-type equivalent rate. [2]: Improvement of the deletion mutant phenotype despite significant difference to wild-type phenotype.

Strain:	K_2TeO_3:	Growth rate:
SL1344	-	0.40 ± 0.05
	+	0.46 ± 0.01
Δ*hmpA*	-	0.44 ± 0.11
	+	0.23 ± 0.03
Δ*hmpA* p*hmpA*	-	0.37 ± 0.07
	+	0.52 ± 0.13
Δ*norV*	-	0.34 ± 0.01
	+	0.27 ± 0.02
Δ*nrfA*	-	0.41 ± 0.01
	+	0.34 ± 0.05
Δ*nsrR*	-	0.41 ± 0.02
	+[1]	0.56 ± 0.02
Δ*STM1273*	-	0.37 ± 0.01
	+	0.33 ± 0.01
Δ*STM1273* p*STM1273*	-	0.34 ± 0.09
	+	0.63 ± 0.00
Δ*ytfE*	-	0.44 ± 0.18
	+	0.18 ± 0.05
Δ*ytfE* p*ytfE*	-	0.34 ± 0.05
	+[2]	0.37 ± 0.04

5.4.4 Growth of strains with deletions of genes from the core NO regulon in the presence of potassium tellurite

In order to be able to compare the overall sensitivity of strains to NO and to tellurite, the full set of core NO regulon mutant strains has been tested for its aerobic growth in the absence and presence of tellurite. In addition to the previously described mutant strains of *hmpA*, *norV*, *yeaR* and *ytfE* that have significantly increased sensitivity towards tellurite and hence slower growth, *cstA*, *hcr* and *ygbA* deletions also result in significantly slower growing strains (Figure 23 and Figure 24). The deletions of *ygbA* and *cstA* decrease the growth by 3% and 13% respectively. However, growth of Δ*hcr* in tellurite-containing medium is only at 62.5% of the growth rate when no tellurite is present (Table 26). The difference in growth rates are minimized (Δ*hcr*) or eliminated (Δ*cstA* and Δ*ygbA*) with the introduction of a complementation construct.

Similar to a deletion of *nsrR*, the Δ*cydB* strain grows better in tellurite-containing medium than the parent strain and is significantly less affected by tellurite.

5.4.5 Expression level changes after tellurite exposure

The expression levels of six genes grown in the absence and presence of 0.125 µg x mL^{-1} potassium tellurite are compared. The expression of the tellurite resistance genes *STM1808*, *tehB* and *yeaR* is compared to the levels of the NO detoxification genes *hmpA* and *norV* to find possible correlations in response to tellurite exposure. The expression levels of *nirB* are also analysed as the *nirB* deletion strains has one of the lowest growth rates during tellurite exposure. Since regulation of *hmpA*, *STM1808* and *yeaR* by NsrR has been shown, a response in expression could be linked to NsrR regulation.

The addition of tellurite is done after three hours of growth in aerobic cultures of SL1344 in LB (Figure 25). A delay in growth is noticeable whilst the cells adapt to the change in growth conditions. Growth levels have recovered to the level of the undisturbed culture five hours after tellurite addition. The analysis of the RNA extracted from cells exposed to tellurite shows that the levels of *STM1808* are significantly increased by two to four fold at fifteen, 30 and 60 minutes after tellurite addition (Figure 26).

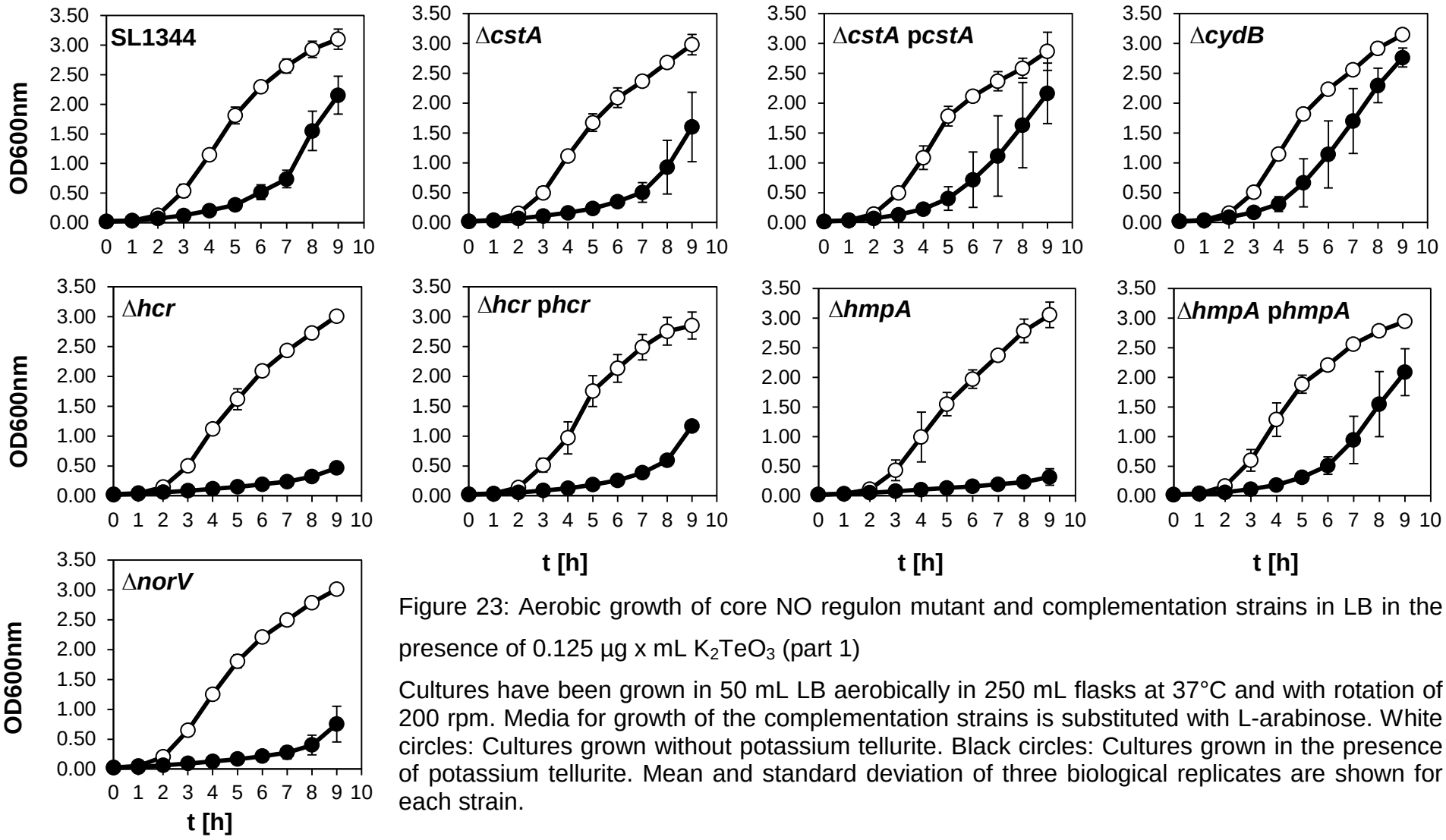


Figure 23: Aerobic growth of core NO regulon mutant and complementation strains in LB in the presence of 0.125 µg x mL K_2TeO_3 (part 1)

Cultures have been grown in 50 mL LB aerobically in 250 mL flasks at 37°C and with rotation of 200 rpm. Media for growth of the complementation strains is substituted with L-arabinose. White circles: Cultures grown without potassium tellurite. Black circles: Cultures grown in the presence of potassium tellurite. Mean and standard deviation of three biological replicates are shown for each strain.

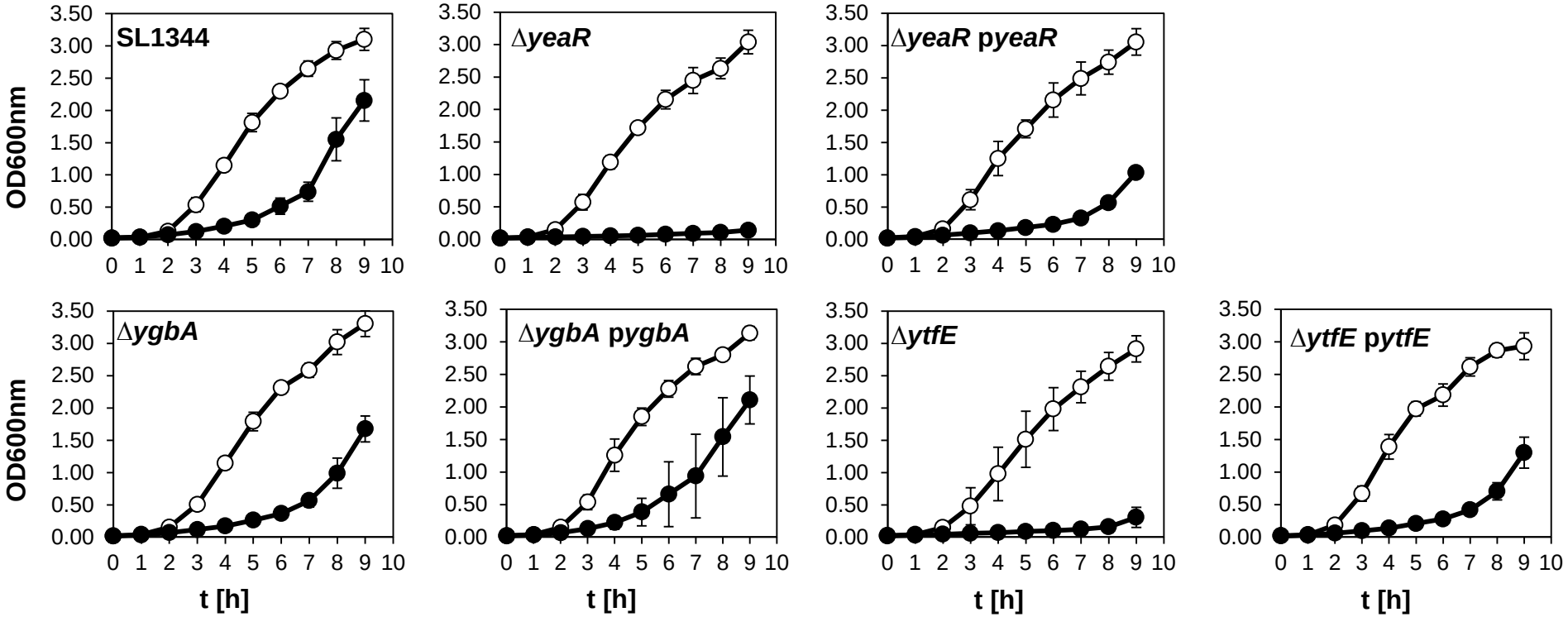


Figure 24: Aerobic growth of core NO regulon mutant and complementation strains in LB in the presence of 0.125 µg x mL K_2TeO_3 (part 2)

Cultures have been grown in 50 mL LB aerobically in 250 mL flasks at 37°C and with rotation of 200 rpm. Media for growth of the complementation strains is substituted with L-arabinose. White circles: Cultures grown without potassium tellurite. Black circles: Cultures grown in the presence of potassium tellurite. Mean and standard deviation of three biological replicates are shown for each strain.

Table 26: Growth rates of core NO regulon mutant and complementation strains grown in the absence (-) and presence (+) of 0.125 µg x mL^{-1} K_2TeO_3 aerobically in LB with shaking at 200 rpm at 37°C.
Growth rates have been calculated using the data of a minimum of three biological replicates and evaluating the growth between 3 and 7 hours into the experiment. No significant differences for growth rates in the absence of potassium tellurite in comparison to wild-type have been detected. Grey shading indicates a significant difference between growth rates in comparison to the respective wild-type rate. [1]: Significantly increased growth rate in comparison to wild-type equivalent rate. [2]: Improvement of the deletion mutant phenotype despite significant difference to wild-type phenotype.

Strain:	K_2TeO_3:	Growth rate:
SL1344	-	0.40 ± 0.05
	+	0.46 ± 0.01
Δ***cstA***	-	0.39 ± 0.05
	+	0.34 ± 0.02
Δ***cstA*** **p*cstA***	-	0.39 ± 0.04
	+	0.50 ± 0.12
Δ***cydB***	-	0.41 ± 0.03
	+[1]	0.57 ± 0.05
Δ***hcr***	-	0.40 ± 0.02
	+	0.25 ± 0.02
Δ***hcr*** **p*hcr***	-	0.40 ± 0.04
	+[2]	0.37 ± 0.03
Δ***hmpA***	-	0.44 ± 0.11
	+	0.23 ± 0.03
Δ***hmpA*** **p*hmpA***	-	0.37 ± 0.07
	+	0.52 ± 0.13
Δ***norV***	-	0.34 ± 0.01
	+	0.27 ± 0.02
Δ***yeaR***	-	0.37 ± 0.05
	+	0.17 ± 0.03
Δ***yeaR*** **p*yeaR***	-	0.36 ± 0.04
	+[2]	0.30 ± 0.02
Δ***ygbA***	-	0.41 ± 0.01
	+	0.40 ± 0.06
Δ***ygbA*** **p*ygbA***	-	0.40 ± 0.06
	+	0.47 ± 0.09
Δ***ytfE***	-	0.44 ± 0.18
	+	0.18 ± 0.05
Δ***ytfE*** **p*ytfE***	-	0.34 ± 0.05
	+[2]	0.37 ± 0.04

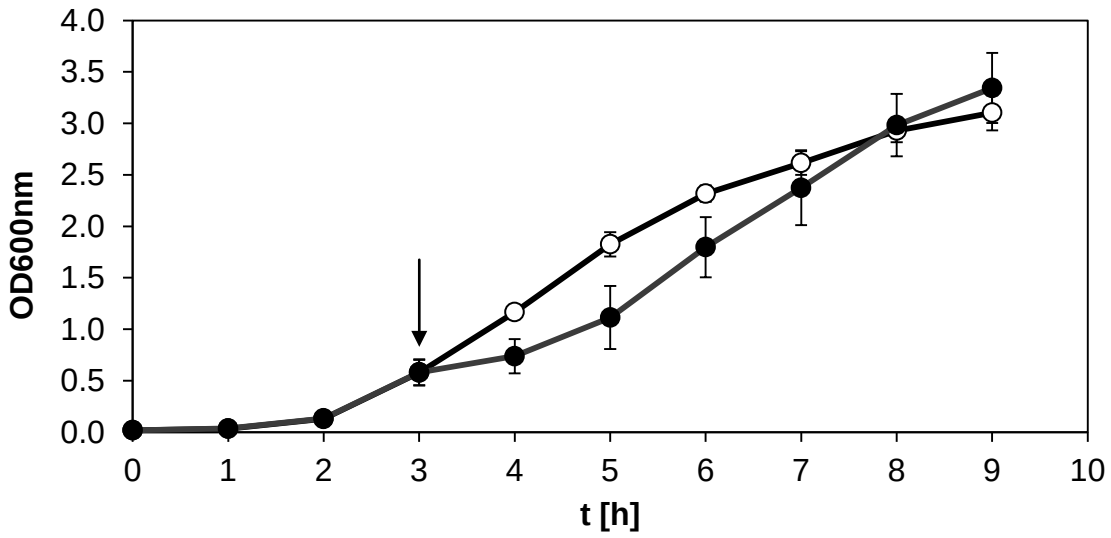


Figure 25: Growth of SL1344 strain with and without addition of 0.125 µg x mL^{-1} potassium tellurite.

White circles show the growth of SL1344 in LB under standard conditions without any tellurite present. Black circles show the growth of SL1344 with an addition of 0.125 µg x mL K_2TeO_3 at three hours indicated by the arrow. RNA extraction is performed before and fifteen, 30 and 60 minutes post tellurite addition. The mean of four biological replicates with standard deviation is shown.

The levels of *tehB* and *yeaR* are also significantly increased at fifteen minutes, but struggle to reach the two-fold cut-off. The levels decrease further with the later time points, although *yeaR* levels are still significantly different to levels prior to tellurite exposure. The levels of *hmpA* do not reach the two-fold threshold and are minimal throughout the time course of the experiment. The other NO detoxification gene of interest, *norV*, does not significantly increase in expression after fifteen, but significantly increases to nearly six-fold after 30 minutes. The level declines to four-fold when the 60 minute time point is reached. *NirB* RNA levels significantly increase to eight-fold at fifteen and 30 minutes and peak at ten-fold after 60 minutes.

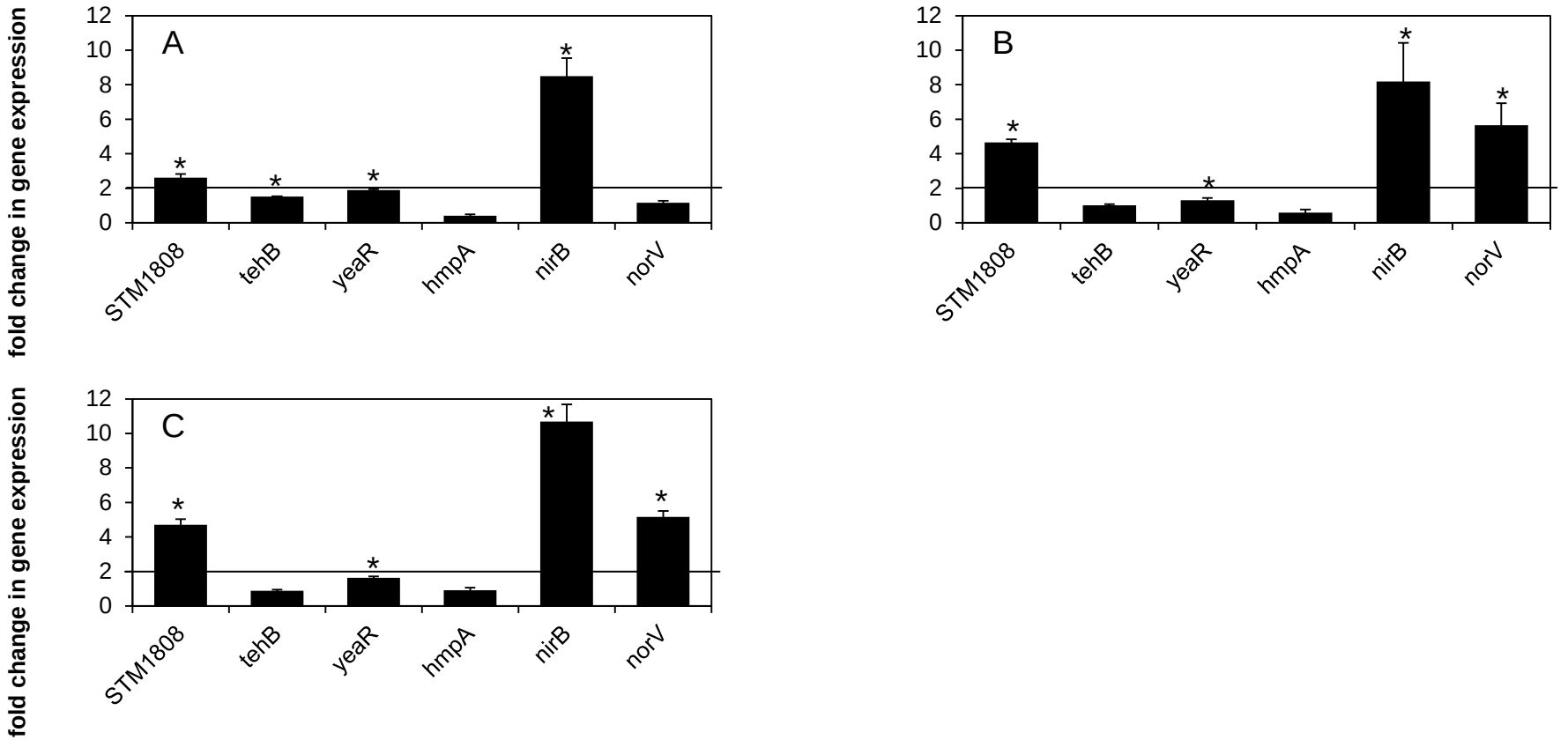


Figure 26: Changes in expression levels of assorted genes after tellurite addition.
Expression levels of *STM1808*, *tehB*, *yeaR*, *hmpA*, *nirB* and *norV* genes fifteen (A), 30 (B) and 60 minutes (C) post the addition of 0.125 µg x mL^{-1} K_2TeO_3, normalised to *ampD* expression levels and to the values obtained at 0 min (i.e. before the addition of tellurite): A 1.5 fold cut off has been applied. Values presented are the mean of four biological replicates with standard deviations. An asterisk indicates a p value below 0.05 as determined by the t test by comparison of expression levels at specific time point with levels at 0 min.

5.5 Discussion

The aim of this chapter has been to determine the contribution of putative tellurite resistance and NsrR-regulated genes to tellurite resistance. Further, the aim is to clarify whether a correlation between tellurite and NO resistance exists. The chapter also aims to investigate the changes in gene expression of tellurite resistance and NO detoxification genes.

This study has shown that the gene products of *yeaR* and *STM1808*, despite previous hypotheses in the literature, do contribute to tellurite resistance in *S.* Typhimurium (Karlinsey et al, 2012). Even though the homology to other *tehB* genes might not be supportive of their protective role during tellurite exposure, the results clearly show that even a single deletion has significant impact on the growth of the strain. The biggest contribution to the sensitivity of a triple deletion strain comes from the deletion of *tehB*. However, the deletion of all three genes further delays the growth. The correlation to the production of ROS might also explain why *Salmonella* has three of these genes rather than just the *tehAB* operon and the *yeaR* gene like *E. coli*. *Salmonella* pathogenicity relies on the survival within macrophages. The two main groups of antibacterial stresses encountered during this process are ROS and RNS. Any additional mechanisms that allow detoxifying either of these provide a competitive advantage during the infection process. Therefore, the evolutionary development of tellurite resistant mechanisms that detoxify hydroxyl radicals from tellurite reduction are also valuable for survival in the presence of ROS and hence during pathogenicity.

The addition of DTT scavenges any ROS present and eliminates any effect ROS have on bacterial growth. The recovery of growth of the tellurite deletion strains in the presence of DTT hence highlight that the deletion of putative and known tellurite resistance genes affects the ability to deal with the toxicity of the ROS generated during the detoxification of tellurite. ROS are also generated in the presence of NO when the generation of free Fe(II) supplies the Fenton reaction, leading to hydroxyl radical formation. Therefore, the link between the tellurite and NO sensitivity of tellurite deletion strains is proposed to originate from the inability of deletion strains to sufficiently detoxify ROS.

Previous reports that NarG and NarZ function as aerobic tellurite reductase have not been confirmed (Avazeri et al, 1997). The expression of *narG* has been shown for anaerobic conditions and therefore, a deletion of *narG* would not cause any changes in expression levels in the tellurite growth experiment (Rowley et al, 2012). This is reflected by the lack of significant growth delay displayed by the Δ*narG* mutant strain. The study by Avazeri *et al.* has been performed using purified protein and therefore any regulatory impacts have been eliminated. It might hence be possible that NarG functions as a tellurite reductase, but such observation has not been made in this study. NarZ, however, has been shown to be expressed during aerobic growth (Spector & Kenyon, 2012). The results presented here though do not support the findings of the reductase activity assays as the deletion of *narZ* does not have any effect on the growth of the mutant strain. The physiological relevance of NarG and NarZ as tellurite reductases is doubtful. As shown here, during aerobic conditions where *narZ* expression is present, no influence on tellurite sensitivity is observed for *S*. Typhimurium. Aerobically, where no NarG expression is expected, no influence on tellurite resistance is observed. Anaerobically, the study by Avazeri *et al.* has not found any enzyme activity. In order to be able to clarify whether the discrepancies stem from differences between *E. coli* and *S*. Typhimurium, a comparative study between these two bacteria is needed. Only when all three nitrate reductase operons are deleted, does *S*. Typhimurium show a significantly reduced growth of 95% with tellurite present.
The two investigated nitrite reductases NirB and NrfA display stronger sensitivities: A growth reduction by 17% (Δ*nrfA*) and 45% (Δ*nirB*) respectively highlight the potential of nitrite reductases to further support *Salmonella* growth during tellurite exposure, although their gene expression has been attributed to anaerobic rather than aerobic conditions (Rowley et al, 2012).

All genes classified for NO detoxification, either confirmed (*hmpA*, *norV*, *nrfA*, *ytfE*) or putative (*STM1273*), increase the tellurite sensitivity of the respective deletion strains. The effect of deleted *hmpA*, *STM1273* and *ygbA* has been reverted with the appropriate complementation constructs, validating the phenotypes observed. When NsrR is deleted, decreased tellurite sensitivity is observed: This indicates a link between NO detoxification mechanisms under

the regulation of NsrR and the toxicity of tellurite. It is hereby proposed that the connection between NO and tellurite toxicity lies in direct and indirect generation of ROS. Disruption of [Fe-S] clusters by both compounds fuels the Fenton reaction that results in ROS production. In addition, ROS are generated when tellurite is being reduced to yield insoluble Te^0. As a result, tellurite resistance mechanisms could benefit *S*. Typhimurium during NO exposure and NO detoxification mechanisms could minimize the toxicity of tellurite exposure. This is also reflected in the sensitivity of the *ytfE* deletion strain: As an [Fe-S] repair enzyme, YtfE is needed during NO and tellurite reactivity with [Fe-S] clusters to maintain protein functionality and to reduce the amount of free iron within the cell.

The core NO regulon genes all influence the tellurite sensitivity when deleted and thus further strengthen the hypothesis that tellurite and NO detoxification are linked. Deletion of cydB shows decreased sensitivity, similar to NsrR and it remains to be determined what the role of this cytochrome *d* ubiquinol oxidase subunit is during tellurite resistance. A previous study using *E. coli* has not found any changes in sensitivity when *cydB* is deleted (Borsetti et al, 2005). In 2000, however, tellurite reduction via the respiratory chain has shown to involve several cytochrome oxidases (Trutko et al, 2000). Expression of CydB is at the highest level during microaerobic conditions, but increased expression is observed an- and aerobically when NO is present (Govantes et al, 2000; Pullan et al, 2007). As a result, the levels of CydB might not be high within the cell during aerobic cell growth and this could eliminate interference of tellurite with its enzyme activity. If the level of affinity of CydB towards an interaction with tellurite is higher than of other cytochrome oxidases, a deletion of *cydB* during aerobic growth would not have much impact on the growth of the cells.

The expression level changes of tellurite resistance genes are of particular interest in this study: To further investigate a possible correlation between NO and tellurite defence, changes in expression levels of NO detoxification genes *hmpA* and *norV* in response to tellurite are compared to level changes of *STM1808*, *tehB* and *yeaR*. In addition, the gene *nirB* is included as a deletion strain displayed major tellurite sensitivity.

At the first time point, all three tellurite resistance gene levels have increased significantly, indicating a response to the tellurite addition. The levels of *STM1808* RNA further increase at the other time points, whereas *yeaR* and *tehB* levels decrease later on. This indicates possible differences in regulation of *STM1808* in comparison to *tehB* and *yeaR*. The signal ratios of *STM1808* mRNA between an *nsrR* deletion and a 14028 parent strain are equally increased compared to *yeaR-yoaG* in a previous study (Karlinsey et al, 2012). A direct link to NsrR regulation, however, has not been confirmed as *hmpA* levels failed to rise at the time points where cells for RNA extraction have been sampled. A late increase of *norV* RNA levels at 30 and 60 minutes indicates a delayed regulatory effect of tellurite on *norV* gene expression. The expression levels of *nirB* drastically increase to eight-fold after fifteen minutes and peak at ten-fold after one hour. Together with the sensitivity data, this indicates that *nirB* plays an important role during tellurite response: A rapid and prolonged increase in gene expression would allow protection against tellurite toxicity. Given the fact that NirB functions as a nitrite reductase, it could function in the reduction of tellurite to tellurium rather than protection against oxygen radical formation and toxicity.

6 Contribution of the *Salmonella* core NO regulon and tellurite resistant genes to macrophage intracellular survival

6 Contribution of the *Salmonella* core NO regulon and tellurite resistance genes to macrophage intracellular survival

6.1 Introduction

During infection, *Salmonella* is able to survive in the intracellular environment of macrophages and epithelial cells. Activation of macrophages is the result of exposure to cytokines such as IFN-γ and to bacterial cell wall components, e.g. LPS (Kalupahana et al, 2005). Activation results in the release of high levels of NO through the induction of iNOS and nitric oxide concentrations of up to 90 µM have been measured (Eriksson et al, 2000; Vazquez-Torres et al, 2000a). LPS on the surface of *Salmonella* is recognized by macrophage receptors, which leads to phagocytosis of *Salmonella* within a phagosome. In addition, *Salmonella* uses its TTSS SPI-1 to induce intracellular uptake. The bacterial vacuole is modified by *Salmonella* to form the tighter SCV. SPI-2 effector proteins then modify the position of the SCV and ensures the acquisition of nutrients from the cytosol to counteracts the depletion of ions by Nramp1 (Figueira & Holden, 2012). This protein is macrophage specific and an ion transporter that removes ions from the SCV to create less favourable growth conditions for bacteria (Forbes & Gros, 2001; Haraga et al, 2008). As described previously, macrophages produce a range of compounds with high bacterial toxicity aiming to eliminate intruding pathogens. Bacteriolytic enzymes are contained within the lysosome, which fuses with the phagosome. Controversy exists whether fusion of the SCV with the lysosome is efficiently prevented by *Salmonella* to avoid exposure to these hydrolytic enzymes (Haraga et al, 2008).

In addition to hydrolytic enzymes, the phagocyte oxidase Phox starts to generate superoxide, hence increasing the oxidative stress levels that result in a high risk of toxicity to invading bacteria sand make Phox an essential component of the innate immune defence.

Lack of the *phox* gene significantly reduces the ability of mice to fight infections by *Salmonella* (Shiloh et al, 1999). In macrophage assays, lack of *phox* leads to a *Salmonella* survival rate of 100% (Vazquez-Torres et al, 2000a).

Furthermore, the exposure of SPI-1 translocator proteins SipBCD in the cytosol triggers the induction of iNOS activity that is responsible for the production of toxic NO (Cherayil et al, 2000). NO reacts with superoxide to form peroxynitrite, further potentiating its toxicity (Bogdan et al, 2000). NO's reactivity inhibits DNA replication and repair, bacterial respiration, synthesis of amino acids and accelerates the creation of ROS due to the release of iron from [Fe-S], feeding

into the Fenton reaction (Fang, 2004; Henard & Vázquez-Torres, 2012; Schapiro et al, 2003; Szabó et al, 1996). SPI-2 has been shown to inhibit the fusion of iNOS-containing vesicles with the SCV, but further specifics of the mechanism are unknown (Chakravortty et al, 2002). In addition, a connection of the nitrite transporter NirC and decreased NO production has been proposed (Das et al, 2009). Channelling of nitrite into the bacterial cytoplasm decreases the chances of auto-oxidation of nitrite yielding NO. Lower NO levels have less of an inhibitory effect on the SPI-2 effector protein SpiC. SpiC is a potent inhibitor of the IFN-γ-triggered JAK/STAT pathway that induces iNOS activity. Less NO therefore leads to inhibition of phosphorylation of the JAK/STAT pathway component STAT-1 by SpiC and prevents induction of iNOS.
The release of antibacterial compounds occurs in a staggered manner to mount a continuous antimicrobial defence (Vazquez-Torres et al, 2000a). The onset of ROS release has been shown to occur one to two hours after the formation of the SCV (Tsolis *et al.*, 1995; Vazquez-Torres *et al.*, 2000). It takes up to eight hours for the nitrosative burst to take place that involves the production of nitric oxide by iNOS.

The addition of the cytokine IFN-γ to the culture medium allows the stimulation of cell culture macrophages prior to the exposure to the surface LPS on bacteria. This knowledge has allowed the systematic use of activated and non-activated macrophages to create different experimental conditions. Using this model, it is possible to compare the uptake of bacteria as well as their survival within macrophages at different time points. Activated macrophages release NO and other RNS shortly after the uptake of the bacteria during the experiment rather than after an eight hour delay using non-activated cells. Therefore, two different scenarios are possible: First, uptake of bacteria into non-activated cells is followed by the oxidative burst at two and nitrosative burst at eight hours. Second, bacterial uptake into activated cells is shortly followed by the release of NO as part of the nitrosative burst in activated macrophages. The latter excludes any priming of gene expression changes in response to ROS that allows for an adaptation to RNS exposure occurring at a later stage.
In comparison to the anaerobic NO sensitivity experiment of the previous chapter, several factors are different. Release of ROS and RNS triggers the

formation of new radical species that are not present when NO is added to anaerobically growing bacterial cultures. The previous assay also relies on a minimal medium that does not mimic the SCV nutrient composition. Therefore, the environment in the SCV is more complex than the conditions of the NO sensitivity assay.

RNS-specific mechanisms are most important during the bacterial exposure to activated macrophages where the RNS release occurs shortly after bacterial uptake, allowing less time for the adaptation of gene expression. This allows for the comparison between those two different conditions and the evaluation on the importance for genes primarily during NO detoxification in activated macrophages or for defence against radicals produced during the reaction of ROS with RNS.

6.2 Aim

The aim of this study is to investigate the intracellular survival of the core NO regulon deletion strains that display significant increase or decrease of NO sensitivity during anaerobic NO exposure. In addition, the tellurite resistant mutant strains are also included as they show significantly increased NO sensitivity. The bacterial cfu from within resting and activated macrophages are compared to identify the significance of early and late NO exposure for bacterial intracellular survival.

6.3 Methods

Bacterial cells are grown overnight in shaking LB cultures at 37 °C. The optical density at λ = 600nm is measured and the bacterial cfu x mL^{-1} is calculated using the following formula: An OD_{600nm} of 1 corresponds to 1.2 x 10^9 cfu x mL^{-1}. Continuous culturing of RAW264.7 cells follows the protocol in 2.14. Briefly, cells are maintained in DMEM with the addition of 5% FBS, L-glutamate and Penicillin/Streptomycin at a temperature of 37 °C and at a CO_2 concentration of 5%. Sub-culturing is performed once a confluency of 75-80% is reached and a confocal microscope is used to evaluate cell morphology and health. Seeding of macrophages is performed 16 hours and 30 hours prior to the addition of bacteria to the culture medium for non- and activated macrophages, respectively. A MOI of 10 is used for both experimental setups. Activation of

macrophages is achieved by the addition of 100 units of IFN-γ to the culture medium 20-22 hours before bacterial addition. After two hours of bacterial exposure, extracellular bacteria are washed off and any residual bacteria killed with the antibiotic Gentamicin. Bacterial numbers are assessed after two and ten hours to allow for the comparison of cfu after uptake into and invasion of macrophages in comparison to survival cfu after exposure to RNS after the nitrosative burst at eight hours. For activated macrophages, the nitrosative burst occurs sooner and therefore exposes bacteria to RNS shortly after uptake. Harvesting bacteria from macrophages is achieved by rupturing macrophages at the given time points by using a solution of PBS containing SDS.

6.4 Results

6.4.1 Intracellular survival in non-activated macrophages

Seven of the eleven tested strains have been found to have significantly decreased survival in comparison to the wild-type strain when the cfu changes between two and ten hours are compared (Figure 27). With decreases by over 80%, Δ*STM1808* and Δ*ygbA* display the highest significant level of sensitivity towards the intracellular environment. With values ranging at 25% of wild-type levels, Δ*cydB*, Δ*STM1808 tehB yeaR* and Δ*tehA* also show major, significant decreases in survival. Also significantly decreased are the cfu of Δ*tehB* (by 28%) and Δ*yeaR* (by 38%). No accumulative effect of a multiple deletion of tellurite resistance genes has been observed as the levels of Δ*STM1808 tehB yeaR* are similar to the cfu changes of Δ*STM1808*. All mutant strains with deletions of single or multiple tellurite resistance genes are significantly affected in their intracellular survival.

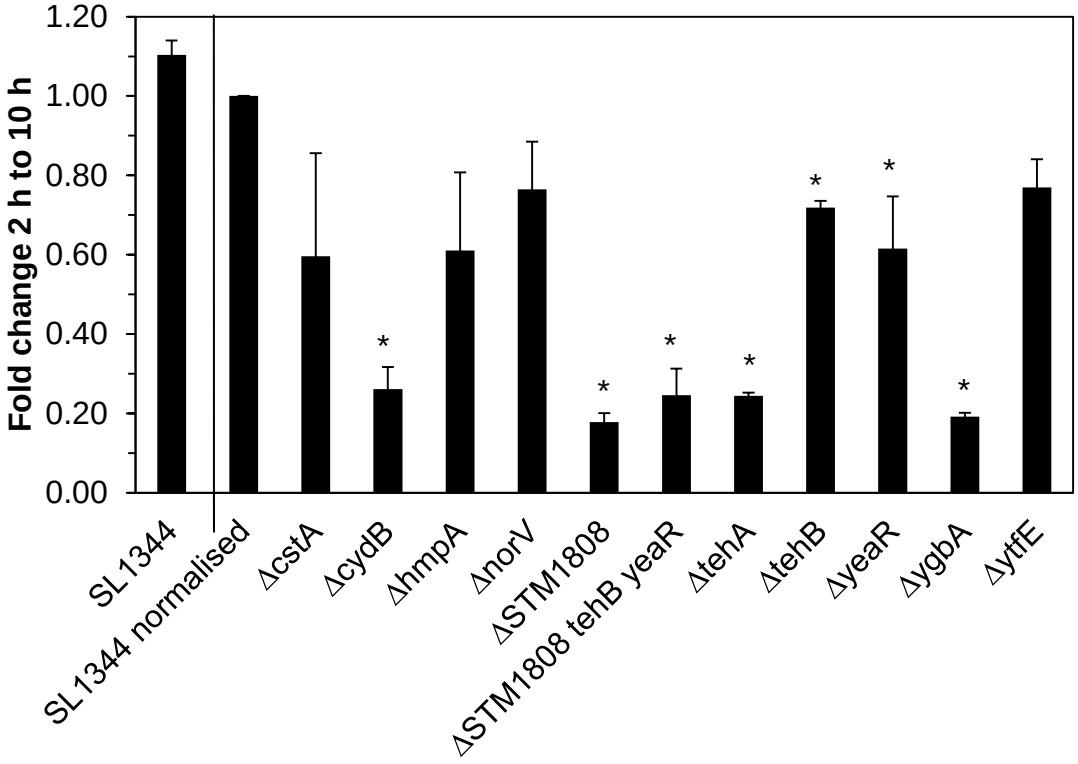


Figure 27: Bacterial intracellular survival of tellurite and NsrR-regulated gene deletion strains between two and ten hours after exposure to non-activated RAW264.7 macrophages.

The wild-type fold-change is shown on the left. On the right, values of deletion strains have been normalised to the bacterial cfu of SL1344 and are shown as a percentage of the wild-type fold change. The values represent a minimum of two biological replicates with three technical replicates each. The error bars show the standard deviations. Asterisk indicates significant differences to wild-type values with $p<0.05$, according to the t test.

Functional components of bacterial respiration, represented by CydB, are also a key element for bacterial pathogenesis. The fold changes in cfu observed for Δ*hmpA* are at 60% of the wild-type fold-change and hence at the same level as shown for Δ*cstA* and Δ*yeaR*. However, the change in intracellular bacterial load is not significant for Δ*cstA* and Δ*hmpA.* Δ*norV* and Δ*ytfE* also have shown a decrease in intracellular cfu, but again this deviation from the wild-type phenotype has not been significant. Possible factors influencing the statistical significance are the high variations between results and the small sample sizes used.

All in all, deletion of tellurite resistance and cytochrome oxidase genes leads to significantly decreased intracellular survival of *S*. Typhimurium within non-activated macrophages. Lower intracellular survival has been observed for known NO detoxification genes, but the effect has not been significantly different from the wild-type phenotype.

6.4.2 Intracellular survival in IFN-γ activated macrophages

Activation of macrophages changes the profile of significantly decreased fold changes of deletion strains. Of the eleven strains tested, seven are significantly different to wild-type (Figure 28). The general decrease of intracellular survival peaks at 56% (Δ*norV*) and 54% (Δ*STM1808 tehB yeaR*). The survival of Δ*hmpA* and Δ*ytfE* significantly drops to 50% and less. The deletion of either *cstA* or *tehA* decreases the fold change by 30%. A similar decrease is observed for Δ*STM1808* and Δ*tehB*; however, the decreases are not significantly different from wild-type. Changes by less than 20% are observed for Δ*cydB*, Δ*yeaR* and Δ*ygbA*, but only the result of Δ*yeaR* has been statistically significant.

This study has confirmed the importance of *hmpA* and *norV* genes for the detoxification of NO. Exposure of single deletion strain to activated macrophages results in significantly decreased intracellular survival. Cfu are down by 50% and more when the bacterial cells are exposed to NO shortly after uptake into macrophages.

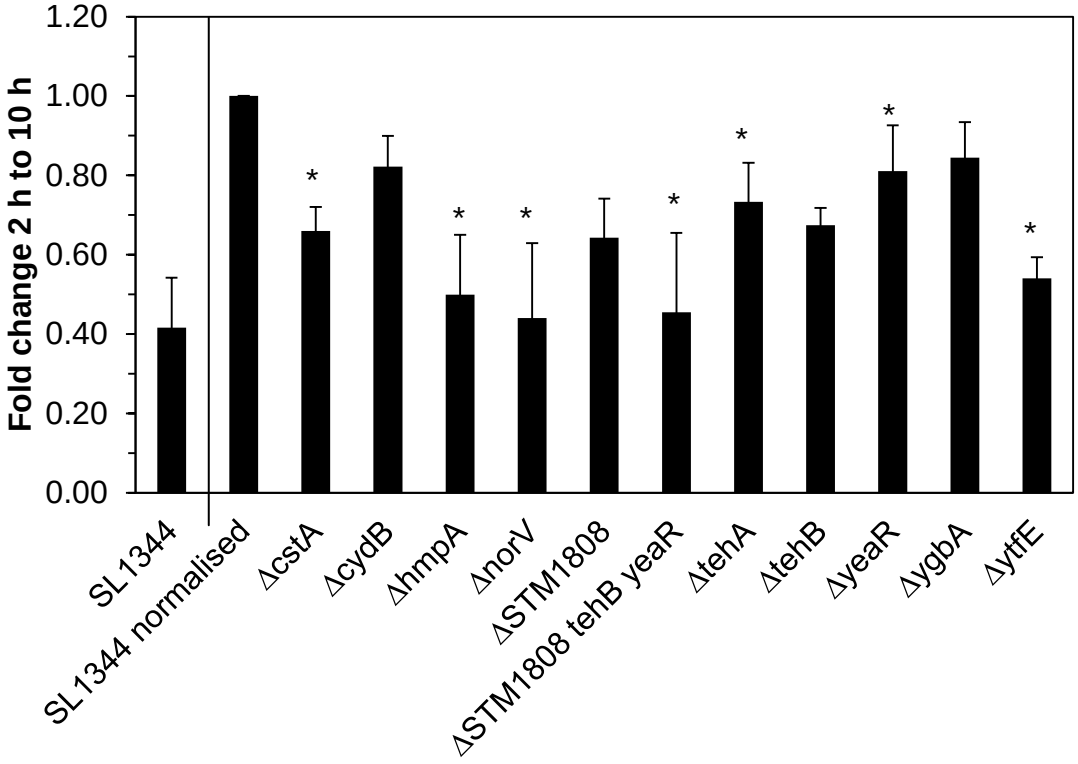


Figure 28: Bacterial intracellular survival of tellurite and NsrR-regulated gene deletion strains between two and ten hours after exposure to activated RAW264.7 macrophages.

The wild-type fold-change is shown on the left. On the right, values of deletion strains have been normalised to the bacterial cfu of SL1344 and are shown as a percentage of the wild-type fold change. The values represent a minimum of two biological replicates with three technical replicates each. The error bars show the standard deviations. Asterisk indicates significant differences to wild-type values with $p<0.05$, according to the t test.

It also shows that the deletion of either does not result in a significant difference to wild-type survival when the NO exposure is delayed in non-activated macrophages. This indicates that *hmpA* and *norV* are important for fast NO detoxification as it is essential for survival in activated macrophages.

6.5 Discussion

The *S*. Typhimurium *hmpA* deletion strain has repeatedly been shown to have a lower survival rate in activated macrophages and a reduced virulence in mice and these observations fit to the results of this study (Bang et al, 2006; Gilberthorpe et al, 2007; Karlinsey et al, 2012; Stevanin et al, 2002; Stevanin et al, 2007). Deletion of *norV* in an *E. coli* strain does not cause any changes in intracellular survival in mouse macrophages or in mouse virulence (Pullan et al, 2007). In *S*. Typhimurium, NorV has been shown to provide protection against macrophage killing, similar to the results observed in Figure 28 (Baptista et al, 2012).

A similar pattern of significantly decreased intracellular survival is observed for Δ*cstA* and Δ*ytfE*, indicating that the [Fe-S] cluster repair performed by YtfE is of particular importance in rapid NO response. The importance of YtfE for pathogenicity has already been shown for *S*. Typhimurium and *H. influenzae*, where *ytfE* deletion decreases virulence in mice (Harrington et al, 2009; Karlinsey et al, 2012; Kim et al, 2003). In addition, *cstA* might have a similar role to *ytfE* or the NO detoxifying genes *hmpA* and *norV*. A previous study has already highlighted that deletion of *cstA* decreases the virulence of *S*. Typhimurium in *C. elegans* (Tenor et al, 2004). However, the virulence assay model using *C. elegans* is lacking nitrosative stress as no nitric oxide is produced by the nematode. Therefore, CstA could be a virulence factor, particularly important under acute NO stress.

When the oxidative burst is proceeding the nitrosative burst after bacterial uptake in non-activated macrophages, the single deletion strains of *STM1808*, *tehA*, *tehB*, *yeaR* and *ygbA* all show significantly decreased survival in comparison to wild-type levels. The combination of deletions of three tellurite resistance genes has not further decreased the poor intracellular survival in

non-activated, but significantly increases sensitivity to the intracellular environment in activated macrophages. The results underline the observation of a previous study, where Δ*STM1808* infection of mice results in significantly decreased bacterial burden of spleen and liver (Karlinsey et al, 2012). A previous study in *H. influenzae* has found a decrease in virulence of Δ*tehB* when the deletion strain is used in a rat infection model (Whitby et al, 2010). No record of using a *tehA* deletion strain has been found. Δ*yeaR* and Δ*STM1808 tehB yeaR* are significantly more sensitive in this study under both conditions, although a mice infection study has not found any significant difference in bacterial burden of Δ*yeaR* (Karlinsey et al, 2012).

Overall, the beneficial effect of tellurite resistance genes on intracellular survival and partially for infectivity has been shown. A comparison of results from this study with the results of Karlinsey *et al.* can be seen in Table 27.

A very strong decrease of survival is observed for Δ*ygbA* in non-activated macrophages. Such an increased sensitivity of the deletion strain is not observed in the mice virulence study (Karlinsey et al, 2012). Deletion of *cydB* also only results in significantly decreased survival in non-activated macrophages. Previously, Δ*cydA* has shown decrease murine virulence, but no change in invasiveness of chicken liver cells has been observed (Turner et al, 2003). In a lung infection assay using a *M. tuberculosis cydC* deletion strain, a decreased bacterial burden is observed (Shi et al, 2005). Transposon mutant assays using murine macrophages and BALB/c mice have found that a functional *cydB* gene is important for survival, but has no significant effect for the virulence in mice overall (Chan et al, 2005). Furthermore, transposon insertions into *cydA* decrease the fitness of *S*. Typhimurium during cattle and mice infection (Chaudhuri et al, 2013). These results indicate that the cytochrome oxidases play an important role during infection, although further details remain to be elucidated.

Several approaches to assess fitness of mutant strains on a larger scale exist. Signature-tagged mutagenesis allows the comparison of the survival of transposon mutant strains *in vitro* with survival in an infection model. This technique has been key to identify SPI-2 (Shea et al, 1996), however, the number of unique tags used for probe hybridization is limited.

Table 27: Comparison of fold-changes and their significance of tellurite and NsrR-regulated gene deletion strains between two and ten hours in non- and activated RAW264.7 macrophages.

Results from the Karlinsey *et al*., 2012 study regarding bacterial burden in liver and spleen of C3H/HeN mice (Nramp1^{+}) are shown. (↓) indicates decreased cfu, whereas (↔) indicates no phenotypic change to wild-type results. (N. D.) indicates that this phenotype has not been determined in the study. Asterisks indicate significant changes to respective wild-type values.

Strain:	**Karlinsey *et al*., 2012**	**Non-activated RAW264.7:**	**Activated RAW264.7:**
Δ***cstA***	N. D.	↓	↓*
Δ***cydB***	N. D.	↓*	↓
Δ***hmpA***	↓*	↓	↓*
Δ***norV***	N. D.	↓	↓*
Δ***STM1808***	↓*	↓*	↓
Δ***STM1808 tehB yeaR***	N. D.	↓*	↓*
Δ***tehA***	N. D.	↓*	↓*
Δ***tehB***	N. D.	↓*	↓
Δ***yeaR***	↔	↓*	↓*
Δ***ygbA***	↓	↓*	↓
Δ***ytfE***	↓*	↓	↓*

In addition, the focus of results often remains on negatively selected strain, i.e. that are less fit than the wild-type strain (Chaudhuri et al, 2013). TraDIS (transposon directed insertion site sequencing) has been developed to allow positive and negative selection of mutants and identification of genomic regions next to transposon sites is achieved using Illumina sequencing (Langridge et al, 2009b). A recent study includes over 7,700 transposon mutant strains and highlights that disruption in several of the core NO regulon genes impacts on the fitness during animal infection models in chicken, pigs, cattle and mice (Chaudhuri et al, 2013). *CstA* is only required in pig infection, whereas an effect of *hmpA* is only seen during the infection of cattle. No effect of *cydB*, *hcr*, *norV*, *ygbA*, *yoaG* or *ytfE* has been reported. However, interestingly, transposon insertions in *tehB* result in reduced fitness within chicken, pigs and cattle. Unfortunately, no report about *yeaR* or *STM1808* has been found in the supplementary data as it would be interesting to know whether the other tellurite resistance genes have a similar effect on survival fitness.

The mixture of ROS and RNS created within non-activated macrophages during the intracellular phase of *Salmonella* infection results in an increased radical production through the Fenton reaction. As a result, bacteria are exposed to both ROS and RNS within the time frame of the experiment. In contrast, exposure to NO, other RNS and ROS in activated macrophages occur at once, but allows for a longer recovery time until the ten hour time point. Therefore, it has been concluded that there are two sets of genes important for intracellular survival: 1) enzymatic NO detoxification and NO damage repair genes and 2) genes that allow detoxification of a combination of nitrogen and oxygen radicals. When the exposure to NO is imminent, e.g. in activated macrophages, the gene products of *hmpA*, *norV* and *ytfE* are needed to detoxify NO and to repair proteins directly affected by NO. Strains significantly affected in non-activated macrophages have deletions in e.g. tellurite resistance genes and hence are predicted to be more sensitive due to radicals produced in the Fenton reaction after NO exposure or tellurite detoxification.

7 General Discussion

As each chapter has been discussed individually, this general discussion emphasizes the major outcomes of the research and provides hypotheses and suggestions for future studies.

7.1 Context

Salmonella is a globally important pathogen, causing millions of cases of typhoid fever and gastroenteritis in humans each year. Outside of humans, *Salmonella* also infects a range of mammals and birds, where consumption of contaminated meat is a common source of human gastroenteritis. Human fatality rates are increased in immunocompromised hosts, the elderly, young babies and children. Factors such as malnutrition and underlying diseases such as Malaria and HIV further increase the severity of the illness. With increased use and misuse of antibiotics for treatment, a rapid rise in antibiotic resistance has evolved, resulting in more difficult management of the disease. Current Typhoid fever vaccines cannot protect against gastroenteritis strains, nor do they offer lifelong immunity against *S*. Typhi infection. Further limitations include storage conditions and the minimum age for recipients of the vaccine. Measures for supplying clean drinking water and heat treatment of water, vegetables and meat are therefore the most successful means of decreasing *Salmonella* infection rates in areas where infection by *S*. Typhi, *S*. Paratyphi and *S*. Typhimurium are common and where access to vaccines is low.

The first step towards infection is the ingestion of *Salmonella* with contaminated food products or water. The combination of RNS from dietary nitrate and the acidity of the stomach trigger the acid tolerance response and also prime gene expression for the later reoccurring exposure to RNS within macrophages. Further passage through the gastrointestinal tract transport *Salmonella* into the small intestine. To avoid antibacterial metabolites from commensal gut bacteria and detection by the immune system, *Salmonella* uses the SPI-1 proteins to invade enterocytes or channels through M cells to reach the basolateral side of the intestinal epithelium. M cells present *Salmonella* bacteria to macrophages that take them up via phagocytosis. Irrespective of how *Salmonella* reaches the intracellular stage, it modifies the vacuolar compartment to form the SCV. Within the SCV, *Salmonella* encounters exposure to ROS and RNS that are

part of the antimicrobial defence mounted by the immune system. Detoxification of ROS and RNS at this stage is essential to ensure survival and the ability to replicate within the host cell. Three enzymes for NO detoxification have previously been identified for *E. coli* and *S*. Typhimurium, namely HmpA, NorV and NrfA. Tolerance levels of NO and intracellular survival are known to be lower for *E. coli* than for *Salmonella*. This raises the question whether additional NO detoxification mechanisms exist in *Salmonella* that allow it to survive higher NO concentrations. *Salmonella* encounters NO in the stomach after the protonation of nitrite yields NO and other RNS and is further exposed to NO in the SCV. Therefore, NO detoxification is an essential step during *Salmonella* pathogenesis and a better understanding of this process would allow the development of antibacterial drugs or vaccines that can target the enzymes involved in NO defence or accentuate the toxicity of NO.

7.2 Identification of the core NO detoxification regulon

7.2.1 Changes in gene expression during exposure to endogenous NO

As a result of denitrification, NO is produced endogenously by *Salmonella*. Detoxification of nitric oxide is then needed to avoid self-poisoning. Anaerobic growth in a bioreactor under nitrate-sufficiency results in NO-genesis and has been used to compare gene expression levels before and after NO production. 46 genes are significantly up-regulated by a minimum of five-fold. The up-regulation of the NO detoxification genes *hmpA* (flavohaemoglobin), *norV* (flavorubredoxin-type nitric oxide reductase) and *ytfE* (iron-sulphur repair) matches previous reports, stresses the importance of NO detoxification during denitrification and also validates the approach taken (Filenko et al, 2007; Flatley et al, 2005; Justino et al, 2005; Karlinsey et al, 2012; Mukhopadhyay et al, 2004).

The most up-regulated genes, at over 120 fold, are *hmpA* and *narG*. *NarG* encodes for an anaerobically-active nitrate reductase, similar to NarZ. The third nitrate reductase Nap is of higher importance during aerobic growth and is not significantly affected by endogenous NO exposure during anaerobic growth (Rowley et al, 2012). Expression levels of *narZ* are also not significantly

affected under the experimental conditions. Therefore, the results suggest that NarG is the major enzyme for nitrate respiration under the given nitrate-sufficient conditions.

The importance of HmpA during survival within murine and human macrophages as well as during mice infection has been shown in the related literature (Bang et al, 2006; Gilberthorpe et al, 2007; Karlinsey et al, 2012; Stevanin et al, 2002). The high expression levels during endogenous NO exposure have stressed the importance of HmpA's nitric oxide dioxygenase activity, providing further proof that HmpA plays a major role during situations where NO levels are high such as during intracellular survival within macrophages. Groups of genes all regulated by NsrR and FNR, known to be NO-responsive transcriptional regulators, are significantly induced and include *cydAB, hcr*, *hmpA*, *narGHIJK*, *yeaR-yoaG*, and *ygbA*.

The methionine biosynthesis operon has previously been shown to be significantly affected by NO toxicity where three *met* genes are significantly increased (Flatley et al, 2005). The increase in expression of *met* operon genes in this study corresponds with the observation that reactivity of NO with homocysteine results in increased expression of *met* genes to compensate for the loss of homocysteine in the methionine biosynthesis pathway (De Groote et al, 1996; Membrillo-Hernández et al, 1998; Schell, 1993).

The increase in *cydAB* (cytochrome *d* oxidase genes) RNA levels have been reported for *E. coli* and *S. aureus* after NO exposure and have been explained to be a mechanism to compensate for the decreased activity levels of other oxidases (Filenko et al, 2007; Richardson et al, 2006; Stevanin et al, 2000). This is further evidence that NO is affecting bacterial respiration.

Results from other studies have also shown the increased gene expression of *hcr*, *hmpA*, *norV* and *ytfE*, supporting the observations that they play an important role during NO detoxification (Filenko et al, 2007; Hautefort et al, 2008; Karlinsey et al, 2012; Partridge et al, 2009).

7.2.2 Changes in gene expression during exposure to exogenous NO

Exogenous NO is encountered during infection of macrophages, where NO is produced in order to kill invading pathogens. Detoxification of NO is essential to enable *Salmonella* to survive and replicate within macrophages as part of the infection process. The addition of 40 µM NO to anaerobically growing *S*. Typhimurium cells significantly changes the gene expression levels of 139 genes by a minimum of two-fold after ten minutes.

Similarly to the observations made during endogenous NO exposure, the levels of *hmpA*, *norVW* and *ytfE* are also significantly increased as shown in previous studies (Filenko et al, 2007; Justino et al, 2005; Mukhopadhyay et al, 2004). With fold-changes above 135, these four genes are in the group of the top ten genes that see the highest fold changes in expression. Other genes in the group include *STM1808* (putative tellurite resistance gene), *STM1868A* (encoding for a lytic enzyme), *STM1250* (encoding for a putative cytoplasmic protein) and *sitABC* (encoding for an iron transport system). The high expression levels of NO detoxification genes underline the importance of NO detoxification for bacterial survival.

Again, genes regulated by FNR and NsrR significantly increase in expression in response to NO. Apart from *hmpA* and *ytfE*, pyruvate dehydrogenase genes (*aceEF*, *lpdA*, *pdhR*) and putative tellurite resistance genes are most noticeably affected (*tehAB*, *STM1808*, *yeaR*).

As seen with the results of endogenous NO exposure, the bacterial respiratory chain is noticeably affected as expression levels of *cydB* (cytochrome *d* oxidase subunit) and *cyoDE* (cytochrome *o* ubiquinol oxidase subunits) are significantly increased. Another compensatory mechanism to counteract NO toxicity is the increased expression of the DNA repair gene *nrdA* which could potentially decrease DNA damage caused by NO as shown in the related literature (Filenko et al, 2007; Flatley et al, 2005; Justino et al, 2005; Lepoivre et al, 1991; Mukhopadhyay et al, 2004). The interference of NO with metal homeostasis and [Fe-S] cluster formation is also observed. Several iron storage (*bfd*), iron uptake (*sit* operon), siderophore (*iro*, *ent*) and [Fe-S] cluster formation genes (*iscR*, *nifSU*, *sufCDS*) are significantly increased, stressing the importance of balanced iron levels within the cell to decrease the amount of radical production

from free iron through the Fenton reaction and the importance of protein repair for the functioning of the cell (Justino et al, 2005).
The observed gene expression level changes correspond with the reported toxicity of NO. Disruption of protein conformation through reactivity with iron, DNA damage and the relief of gene repression through NsrR and FNR are all confirmed as the corresponding genes are significantly up-regulated.

7.2.3 The core NO regulon

The overlap between up-regulated genes after endogenous and exogenous NO exposure has been determined in order to identify genes that belong to a core NO regulon. Nine genes are up-regulated more than two-fold under both conditions: *cstA*, *cydB*, *hcr*, *hmpA*, *norV*, *yeaR-yoaG*, *ygbA* and *ytfE*, all of which share a number of common regulators such as NsrR and FNR. HmpA, NorV and YtfE have previously been shown to be involved in NO defence through enzymatic detoxification (HmpA and NorV) and protein repair (YtfE). However, very little is known about the contribution of the other proteins during detoxification of NO.

All core NO regulon genes are conserved in the following *Salmonella* strains: *S*. Typhimurium strains LT2, SL1344, DT104 and 14028s; *S*. Typhi strains Ty2 and CT18; *S*. Paratyphi A; *S*. Gallinarum; *S*. Enteritidis PT4 and *S*. Choleraesuis (Silva et al, 2012). In *S*. Paratyphi C, only *hcr*, *norV* and *ygbA* are well-conserved, but the other core NO regulon genes are considered absent as the gene identity is at levels of 85% or below. *CydB* and *yeaR-yoaG* are not well conserved in *S*. Paratyphi B, whereas overall, *cstA*, *cydB*, *hcr*, *norV* and *ytfE* are well-conserved across *S*. Dublin, *S*. Diarizonae, *S*. Arizonae and *S*. *bongori*. *YeaR* is the gene which is least well conserved across these last three genes. In conclusion, *norV, cstA, hcr, ytfE*, *ygbA* and *cydB* are most well-conserved from the core NO regulon genes with an average gene similarity of above 97%. Average values in the range of 90-93% have been determined for *hmpA, yeaR* and *yoaG*.

The carbon starvation protein-encoding gene *cstA* has not previously been linked with NO defence, but a role during nematode infection, where no nitric

oxide is present, has been recognized (Tenor et al, 2004). Increased expression levels of *cydB* have been explained with the increased demand for energy during infection (Hautefort et al, 2008). In addition, nitric oxide inhibits the activity of CydB, making it prone to malfunctioning during NO exposure (Borisov et al, 2004; Stevanin et al, 2000). Hcr functions as an oxidoreductase for Hcp, with a previous NO exposure assay showing the expression of the gene to be increased (Kim et al, 2003). However, its role during infection is not clear as deletions of *hcr* and its operon partner *hcp* increase bacterial survival within murine liver and spleen, although at lower inoculation doses. Also, a *hcp* deletion strain has the same NO sensitivity as the wild-type strain (Karlinsey et al, 2012). This raises the question about the exact role of Hcr in the infection process.

The next two genes on the list of core NO regulon genes have been linked to NO defence for some time: HmpA and NorV can convert NO into less toxic products and are an integral part of NO detoxification of *Salmonella* (Crawford & Goldberg, 1998b; Hausladen et al, 1998; Mills et al, 2008). Alongside *norV* and *hmpA*, *ytfE* expression has also been linked to the presence of NO (Bodenmiller & Spiro, 2006; Gilberthorpe et al, 2007; Justino et al, 2005; Pullan et al, 2007). Its role has been shown to allow for the repair of [Fe-S] clusters (Justino et al, 2007). The strong induction of *hmpA*, *norV* and *ytfE* to a number of NO donor agents strongly supports their role during NO detoxification.

YeaR, *yoaG* and *ygbA* have been less in the focus of interest in studies looking at NO toxicity, although increased gene expression in the presence of NO sources has been reported (Constantinidou et al, 2006; Justino et al, 2005; Mukhopadhyay et al, 2004). The DUF of *yeaR* indicating a tellurite resistance role has not been investigated further, nor any possible function of *ygbA* in NO detoxification or damage repair.

Overall, the expression levels of three well-characterised NO defence genes have been shown to change significantly, alongside a number of genes that have only recently been shown to respond to NO. The further investigation of the NO sensitivity of single deletion mutants is necessary to validate the grouping into the core NO regulon.

7.3 Sensitivity towards NO

The anaerobic NO sensitivities of mutant strains with deletion of known NO detoxification, core NO regulon and tellurite resistance genes are investigated. The results shown here agree with previously published results in that both Δ*norV* and Δ*nrfA* display significantly increased NO sensitivity, showing that NO detoxification is an important process (Mills et al, 2008). The sensitivity of Δ*hmpA* is not significantly increased, supporting its minor role during anaerobic NO detoxification determined by Mills *et al.* in 2008. Deletion of the putative NO reductase STM1273 does not result in any changes in anaerobic NO sensitivity, questioning its gene annotation, at least under the conditions used. Δ*ytfE* displays no significant change in nitric oxide sensitivity, in line with previous observations (Gilberthorpe et al, 2007; Karlinsey et al, 2012; Kim et al, 2003). De-repression of NsrR-regulated genes through deletion of NsrR results in decreased NO sensitivity. NsrR deletion allows continuous expression of NO defence genes such as *hmpA* and *ytfE* that detoxify NO and repair the protein damage caused. The anaerobic nitric oxide sensitivity of Δ*hcr* is not significantly increased. This is possibly due to the influence of nutrient and oxygen availability or functional redundancy. Nutrient and oxygen availability could also explain the results observed for Δ*yeaR* and Δ*ygbA*. A previous study by Karlinsey *et al.* has not detected any aerobic NO sensitivity of *Salmonella* strains lacking either of these genes, but a significant anaerobic NO sensitivity is shown in this study, highlighting that functionality of YgbA and YeaR is influenced by oxygen tension. The increase in sensitivity for both deletion strains is very close to the levels detected for Δ*norV* and hence *ygbA* and *yeaR* play an important role during anaerobic NO defence. Similar sensitivity is also observed for Δ*cstA*, highlighting that the presence of CstA plays an important role during NO exposure.

The deletion of *cydB*, encoding a subunit of a cytochrome *d* oxidase, results in significantly decreased NO sensitivity, suggesting that this particular cytochrome oxidase is not highly affected during *in vitro* NO exposure. A mice infection study has shown that deletion of the other subunit, *cydA*, decreases virulence, indicating the more complex *in vivo* environment causes *S*. Typhimurium to require more functional cytochrome oxidases than during *in vitro* studies where only one stress factor is introduced (Turner et al, 2003).

For the first time, it has been shown that the deletion of single tellurite resistance genes significantly increases NO sensitivity. The link between tellurite resistance genes and NO detoxification has not been suggested before. Deletion of all three tellurite resistance genes results in a strain with severe growth impairments when exposed to NO. Irrespective of the gene annotation, *STM1808*, *tehAB* and *yeaR* play a major role during survival in the presence of NO.

In conclusion, NO sensitivity of deletion strains is dependent on oxygen and nutrient availability. Discrepancies with other studies most likely are the result of aerobic vs. anaerobic growth. Overall, the majority of core NO regulon genes are important during nitric oxide defence. In addition, a contribution of, and functional redundancy between tellurite resistance genes in surviving NO stress has been shown for the first time.

7.4 Sensitivity towards tellurite

NsrR-regulation of the *yeaR* homolog *STM1808* has been previously computationally predicted and after the experiments which determined NsrR regulation of *STM1808* in this thesis have been performed, direct NsrR regulation has been confirmed in the literature (Karlinsey et al, 2012; Rodionov et al, 2005).

Ironically, this is the first study to experimentally investigate the effect of tellurite resistance genes on the tellurite sensitivity of *Salmonella*. A correlation between NO sensitivity of Δ*STM1808* and its tellurite resistance domain has been dismissed in the past and no tellurite sensitivity has been tested (Karlinsey et al, 2012). Single deletion strains are greatly affected by the presence of tellurite and a triple deletion strain struggles to grow even over the time course of nine hours. This study has clearly stressed that all three genes *STM1808*, *yeaR* and *tehB* are important for tellurite resistance, despite any protein truncations of STM1808 and YeaR in the C-terminal domain that might render the SAM-dependent methyltransferase dysfunctional (Karlinsey et al, 2012).

During tellurite reduction to tellurium, ROS are produced that lead to a high level of oxidative stress. Scavenging of ROS through the addition of DTT restores the growth of all three single tellurite deletion strains. This is proof that

the sensitivity of the deletion strains is the result of ROS production and that all three genes function in protection against oxidative stress. The correlation between NO and tellurite toxicity potentially lies in the production of oxygen radicals during tellurite reduction and during NO reactivity with [Fe-S] clusters, and a subsequent release of iron. Therefore, deletion of a tellurite resistance gene would also affect cells growing in the presence of NO as ROS detoxification is impaired. It would appear then that the label of tellurite resistance genes is somewhat misleading and that they are actually stress response genes which respond to damaging free radical stress.

This study has not confirmed previous reports about tellurite reduction by nitrate reductases (Avazeri et al, 1997). During aerobic growth, NarG expression does not take place and a deletion strain does not show any signs of altered tellurite sensitivity. Although NarZ is expressed during aerobic growth, deletion of *narZ* does not impact tellurite sensitivity. The sensitivity of Δ*nirB*, however, is extremely high and Δ*nrfA* also has significantly increased tellurite sensitivity, pointing at a physiological role of these nitrite reductases during tellurite reduction, possible as the reductases might be able to bind and reduce tellurite to tellurium.

There is a strong correlation between NO and tellurite sensitivity of deletion strains of known NO detoxification genes (*hmpA*, *norV*, *nrfA*, *ytfE*). Significant increases in tellurite sensitivity of the respective deletion strains are observed, confirming the hypothesis that NO and tellurite toxicity are linked.

Across the remainder of the core NO regulon genes, Δ*cydB* is less tellurite sensitive than the wild-type strain and the same phenotype is observed for Δ*nsrR*. The other core NO regulon deletion strains are all significantly more tellurite sensitive than the wild-type strain. This further stresses the correlation between NO and tellurite resistance.

The same low concentration of tellurite used for the growth sensitivity assay is also used during the monitoring of expressional changes. RNA levels of *nirB* display the highest fold changes at the three time points and this corresponds to the drastically increased tellurite sensitivity of the respective deletion strain. Fold changes of *norV* and *STM1808* take longer to reach their peaks and for *norV* to breach the two-fold cut-off, but the four-fold changes measured 30

minutes post tellurite addition persist until the 60 minute mark. The fold changes of *hmpA*, *tehB* and *yeaR* do not change more than two-fold after tellurite addition. The strong NsrR regulation known for *hmpA* therefore might not be affected by tellurite. The differences between levels of *STM1808* and *yeaR* indicate that despite high homology of the genes, their regulation under tellurite stress is differing. It has become evident that tellurite exposure causes prolonged changes in gene expression levels, raising the questions whether there is a tellurite-responsive regulator either specific to tellurite or responsive to several toxic metal anions.

The presence of additional mechanisms for ROS detoxification could provide *S*. Typhimurium with a competitive advantage during infection as other pathogenic bacteria such as pathogenic *E. coli* strains only have TehB and YeaR to boost survival under oxidative and nitrosative stress.

7.5 Intracellular survival within non- and activated macrophages

Non-activated macrophages require approximately one hour before ROS are released into the phagosome or the SCV. RNS release takes place eight hours after the uptake of bacteria when no prior activation of macrophages has taken place. Therefore, any bacterial cells that are taken up into the non-activated macrophages encounter ROS release first, followed by NO production at a later stage. Activated macrophages produce NO at a high level shortly after bacterial uptake, therefore omitting the exposure to ROS.

All the tested strains show decreased intracellular survival in both non- and activated macrophages when cfu levels at two and ten hours are compared to wild-type levels. However, the deletion of NO detoxification genes *hmpA*, *norV* or *ytfE* causes non-significant decreases in non-activated murine cells along with the result of Δ*cstA*, whereas the decrease is significant for the results from the activated macrophage cells. These results are consistent with previously published studies showing decreased survival in activated macrophages (Δ*hmpA*, Δ*norV*) and decreased mice virulence (Δ*hmpA*, Δ*ytfE*) (Bang et al, 2006; Baptista et al, 2012; Gilberthorpe et al, 2007; Karlinsey et al, 2012; Kim et al, 2003; Stevanin et al, 2002; Stevanin et al, 2007). Survival within activated

macrophages requires a fast response to the NO released in comparison with the eight hour delay of NO production within non-activated macrophages. Lack of *cstA* has proven to reduce nematode virulence (Tenor et al, 2004). As nematodes do not produce NO to kill pathogens, this screening model is more indicative to highlight virulence factors such as possibly CstA rather than NO detoxifying mechanisms.

On the contrary, the intracellular survival of Δ*cydB*, Δ*STM1808*, Δ*tehB* and Δ*ygbA* is significantly decreased in non-activated macrophages, but not in activated cells. These genes hence are less important for a rapid NO detoxification as observed for *hmpA*, but support the detoxification of ROS and RNS in general. The triple deletion strain Δ*STM1808* Δ*tehB* Δ*yeaR* shows significantly decreased intracellular survival in both experimental conditions, but survival is most drastically reduced in non-activated macrophages. Similar, deletion of *yeaR* results in significantly reduced survival in both conditions, but the reduction is bigger when Δ*yeaR* is subjected to non-activated macrophages.

In conclusion, tellurite resistance genes contribute significantly to intracellular survival and this has partly been reflected in the role of *STM1808* and *tehB* during mice and rat infection in previous work (Karlinsey et al, 2012; Whitby et al, 2010). The poor intracellular survival of Δ*cydB* in non-activated macrophages in this study is reflected by the importance of the *cyd* operon during mice virulence shown previously (Turner et al, 2003). A previous study has listed *cydB* as one of the genes where transposon mutations have led to decreased survival within murine macrophages (Chan et al, 2005).

Although Δ*ygbA* struggles to survive within non-activated macrophages, no effect on mice virulence has been observed previously, and the function of YgbA has not been further elucidated (Karlinsey et al, 2012). NO detoxification and repair genes are shown to be of most importance during NO exposure as the deletion strains are more affected in activated macrophages. Therefore, there are two groups of genes that ensure *S*. Typhimurium survival within macrophages. Firstly, tellurite resistance proteins and cytochrome oxidases are most needed to counteract radical formation from the oxidative and nitrosative burst and to maintain the cellular energy metabolism. Secondly, NO detoxifying

and repair enzymes are needed after the release of NO in order to reduce NO reactivity with proteins and DNA.

7.6 Future work

Most of the core NO regulon and the tellurite resistance genes have only been minimally characterised in *Salmonella* to-date. Therefore, further experiments are needed to provide a more precise function of the proteins during NO detoxification, intracellular survival and overall during infection.

It would be of interest to perform a range of experiments to investigate the time point when each of these proteins is expressed during the course of a macrophage infection study, for example by using GFP-labelling, high resolution microscopy and time lapse techniques. These experiments could provide clarification as to whether the intracellular macrophage environment as such or the nitrosative burst in particular is responsible for the activation of gene expression.

Additionally, an iNOS inhibitor, such as aminoguanidine or L-N^G-monomethyl arginine, could be used to selectively stop the production of NO by iNOS within macrophages (Corbett & McDaniel, 1996; Kim et al, 2003; Stevanin et al, 2002). One of these inhibitors could be used to completely inhibit NO synthesis from the start of the bacterial uptake or to abort NO production shortly after the onset of the nitrosative burst. In comparison with intracellular survival in $iNOS^{-/-}$ and $iNOS^{+/+}$ macrophages, this could help to establish the vulnerability of deletion strains to differing time periods of exposures to NO during intracellular survival.

Infection studies and virulence models

These experiments would also lay the basis for mouse infection studies, either by intraperitoneal injection or oral administration of *S*. Typhimurium for competition assays between wild-type and mutant strains (Bang et al, 2006; Karlinsey et al, 2012). In addition, iNOS-deficient mice serve as a NO-free model. Parameters for observations could be the bacterial burden of organs such as the liver and spleen as previously used for *S.* Typhimurium mice virulence assays (Bang et al, 2006; Karlinsey et al, 2012; Kim et al, 2003).

In contrast to macrophage and mice models, other virulence models exist, e.g. using *C. elegans* as the nematode does not have a nitric oxide synthase it could use to prevent bacterial infection. Such a model would highlight any genes that are of importance from a more general aspect of virulence, i.e. are not specific during encounters with NO. Δ*cstA* has already been tested in a *C. elegans* virulence model and has showed significantly decreased virulence, but the other core NO regulon genes have not yet been tested (Tenor et al, 2004).

Biochemical protein characterisation

With regards to biochemical and biophysical characterisation, these results have opened up a whole range of potential experiments. The crystal structures of several core NO regulon and tellurite resistance proteins have yet not been determined, e.g. for CstA, STM1273, STM1808 and YeaR. A limited number of purifications has been performed that would allow for a biochemical analysis. Possible enzyme activity assays include nitric oxide or tellurite reduction, NO oxygenase activity or [Fe-S] repair. One technique is protein film voltammetry which has been used to determine the NO reductase activity of NrfA (van Wonderen et al, 2008). An electrode replaces the membrane and directly supplies electrons to the protein that forms a monolayer on the electrode (Anderson et al, 2001). The electron transfer is measured and information about enzyme kinetics are obtained using only small quantities (picomole) of purified protein (Heering et al, 1998).

Furthermore, the ligand binding, potential dimerization, potential cofactors such as FAD or NAD(P)H or inhibitors, and absorption spectra to identify e.g. NO-mediated changes in haem groups or rubredoxin domains, using UV/visible, X-ray absorption or electron paramagnetic resonance spectroscopy would be of particular interest for better understanding of the protein functions. As differences between aerobic and anaerobic properties might exist, as it is the case for HmpA, different oxygen levels should be taken into consideration (Kim et al, 1999; Mills et al, 2001).

With previous evidence in the literature that *cst* genes play a role during starvation conditions in *E. coli* and *S. Typhimurium*, further tests to investigate the role of *cstA* during intracellular survival would be useful (Dubey et al, 2003;

Schultz et al, 1988; Spector et al, 1988). It would be of interest to examine whether a *cstA* deletion strain is less fit under starvation conditions, e.g. shows growth deficits in minimal media such as MM5.8 that mimics the low pH of the macrophage environment.

Even though it has been shown that bacterial respiration suffered during NO exposure, deletion of *cydB* in this study shows the opposite effect as a deletion strain is significantly less sensitive to grow in the presence of NO than the parent strain (Fang, 2004). Nevertheless, Δ*cydB* has significantly decreased survival within non-activated macrophages and *cydA* deletion in *S*. Typhimurium reduces the virulence in mice (Turner et al, 2003). Therefore, the influence of CydB could be more directed at the metabolic fitness and hence would have a bigger impact during the more complex *in vivo* conditions. It would be of interest to perform studies looking at NO binding and dissociation with CydAB and other cytochrome oxidases to compare the effect NO has on their enzyme activity.

Similar to *STM1273*, *hcr* deletion does not result in increased NO sensitivity. Aerobic sensitivity of Δ*hcp* to NO has previously been shown and deletion of *hcr* might also lead to increased aerobic NO sensitivity (Karlinsey et al, 2012). A combination mutant of *hcr* and *hmpA* deletion could also facilitate to uncover possible functional overlap between both genes. Whether Hcr has the ability to deoxygenate NO similar to HmpA would need to be established using enzyme activity assays similar to the ones performed for HmpA in previous studies (Gardner et al, 2000; Hausladen et al, 1998).

STM1273 has been annotated as coding for a putative nitric oxide reductase, but no anaerobic NO sensitivity for the deletion strain has been observed. Determining the aerobic NO sensitivity of this mutant would therefore be of interest. A combination mutant lacking *norV*, *nrfA* or *hmpA* as well as *STM1273* might provide information about functional redundancy that could have masked any nitric oxide reductase activity of STM1273. If any NO sensitivity is detected, protein purification to further investigate anaerobic or aerobic NO reductase activity would then be a possible step (Gomes et al, 2002).

Tellurite resistance genes

The tellurite sensitivity assay highlights the ability of STM1808 and YeaR to protect *Salmonella* against tellurite toxicity. Previous work has shown the

importance of conserved histidines for functionality of STM1808, but no further protein characterisation or structural analysis has been performed nor have similar experiments been performed for YeaR (Karlinsey et al, 2012). Sequence alignments of *S*. Typhimurium and *E. coli tehB* with *H. influenzae tehB* indicate a truncation at the C-terminal domain. Therefore, it has been questioned whether STM1808 and YeaR as homologues of *S*. Typhimurium TehB would be able to have any SAM-dependent methyltransferase activity. Protein purification would thus allow further enzyme activity studies, e.g. for methyltransferase activity, and protein crystallization studies to establish the protein structures. Substitution of putatively important residues in YeaR would allow assessing which residues are needed for enzyme activity and comparison with STM1808 could provide information on how similar their function is. Additionally, deleting the C-terminal domain from TehB or inactivating the associated methyltransferase through amino acid substitution could elucidate whether the enzyme function of TehB solely relies on the methyltransferase.

A previous study by Avazeri *et al*. has used purified NarG and NarZ to investigate aerobic tellurite reduction activity. The results shown here do not support their role for aerobic tellurite resistance. Since *narG* expression is limited to anaerobic environments, aerobic tellurite sensitivity assay and enzyme activity studies do not match physiological conditions of NarG activity (Rowley et al, 2012). A residual tellurite reductase activity of a Δ*narG narZ* strain is observed that could be attributed to the aerobically expressed nitrate reductase Nap.

For investigation of NarG contribution to tellurite resistance, a deletion strain would need to be tested during anaerobic growth. Differences between sensitivities of *E. coli* and *S*. Typhimurium strains might exist and this could be investigated via a side-by-side study of corresponding deletion strains.

Deletion of *nirB* highlights that this nitrite reductase is a major component for tellurite resistance. Tellurite reduction assays with purified protein would need to be performed to further investigate the enzyme kinetics of NirB. Additionally, cross-sensitivity of tellurite sensitive strains to other metals would need to be investigated. Similar work has already highlighted links to anions of arsenic and selenium in *E. coli* (Turner et al, 2001; Turner et al, 1992).

It has not been established, how gene expression might be modulated by the presence of tellurite directly. It is assumed that the production of ROS leads to expression changes, but the possibility of a tellurite-sensing regulatory system has not yet been explored. It has been shown here that NsrR is contributing to gene expression regulation, but metal-responsive regulators such as BaeSR, Fur and PhoPQ have not yet been assayed for a response to tellurite (Appia-Ayme et al, 2011; Nies, 1999; Runkel et al, 2013).

7.7 Concluding remarks

The work presented in this study stresses the importance of nine additional genes for the detoxification of NO or the repair of NO damage and shows a link between NO and tellurite sensitivity for the first time. An overview of the phenotypes of the tested strains is given in Table 28 and underlines that functional tellurite resistance also confers an advantage for NO stress and intracellular survival of *S*. Typhimurium.

Although the characterisation of the core NO regulon and tellurite genes still offers a huge potential for future work, this work has set the basis in showing the connection between the resistance to tellurite and defence against NO via the ROS produced as a result of their reactivity and reduction.

Table 28: Overview of phenotypes of core NO regulon, tellurite and NO detoxification deletion mutant strains.
The sensitivity during anaerobic NO exposure and aerobic growth in the presence of tellurite are all significant results. Results of intracellular survival are shown and (n.s.) indicates non-significant results. (↓) and (↑) refer to de- and increased sensitivity of a strain, respectively and (wt) indicates that the wild-type's phenotype has been observed. (n.d.) refers to phenotypes that have not been determined in the studies.

Strain:	**NO sensitivity ($-O_2$)**	**K_2TeO_3 sensitivity ($-O_2$)**	**Intracellular survival: RAW264.7**	**Intracellular survival: RAW264.7 + IFN-γ**
Δ***cstA***	↑	↑	↓ n.s.	↓
Δ***cydB***	↓	↓	↓	↓ n.s.
Δ***hcr***	wt	↑	n.d.	n.d.
Δ***hmpA***	wt	↑	↓ n.s.	↓
Δ***norV***	↑	↑	↓ n.s.	↓
Δ***yeaR***	↑	↑	↓	↓
Δ***ygbA***	↑	↑	↓	↓ n.s.
Δ***ytfE***	wt	↑	↓ n.s.	↓
Δ***STM1808***	↑	↑	↓	↓ n.s.
Δ***tehA***	↑	↑	↓	↓
Δ***tehB***	↑	↑	↓	↓ n.s.
Δ***yeaR***	↑	↑	↓	↓
Δ***STM1808 tehB yeaR***	↑	↑	↓	↓
Δ***hmpA***	wt	↑	↓ n.s.	↓
Δ***norV***	↑	↑	↓ n.s.	↓
Δ***nrfA***	↑	↑	n.d.	n.d.
Δ***nsrR***	↓	↓	n.d.	n.d.
Δ***STM1273***	wt	↑	n.d.	n.d.
Δ***ytfE***	wt	↑	↓ n.s.	↓

8 Appendix

8 Appendix

8.1 Publications

Arkenberg A, Runkel S, Richardson DJ, Rowley G (2011) The production and detoxification of a potent cytotoxin, nitric oxide, by pathogenic enteric bacteria. *Biochem Soc Trans* **39**(6)**:** 1876-1879

Rowley G, Hensen D, Felgate H, Arkenberg A, Appia-Ayme C, Prior K, Harrington C, Field SJ, Butt JN, Baggs E, Richardson DJ (2012) Resolving the contributions of the membrane-bound and periplasmic nitrate reductase systems to nitric oxide and nitrous oxide production in *Salmonella enterica* serovar Typhimurium. *Biochem J* **441**(2)**:** 755-762

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The production and detoxification of a potent cytotoxin, nitric oxide, by pathogenic enteric bacteria

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Abstract

The nitrogen cycle is based on several redox reactions that are mainly accomplished by prokaryotic organisms, some archaea and a few eukaryotes, which use these reactions for assimilatory, dissimilatory or respiratory purposes. One group is the Enterobacteriaceae family of Gammaproteobacteria, which have their natural habitats in soil, marine environments or the intestines of humans and other warm-blooded animals. Some of the genera are pathogenic and usually associated with intestinal infections. Our body possesses several physical and chemical defence mechanisms to prevent pathogenic enteric bacteria from invading the gastrointestinal tract. One response of the innate immune system is to activate macrophages, which produce the potent cytotoxin nitric oxide (NO). However, some pathogens have evolved the ability to detoxify NO to less toxic compounds, such as the neuropharmacological agent and greenhouse gas nitrous oxide (N_2O), which enables them to overcome the host's attack. The same mechanisms may be used by bacteria producing NO endogenously as a by-product of anaerobic nitrate respiration. In the present review, we provide a brief introduction into the NO detoxification mechanisms of two members of the Enterobacteriaceae family: *Escherichia coli* and *Salmonella enterica* serovar Typhimurium. These are discussed as comparative non-pathogenic and pathogenic model systems in order to investigate the importance of detoxifying NO and producing N_2O for the pathogenicity of enteric bacteria.

Enteric bacteria, NO and human health

Enteric bacteria find their natural habitat in the intestines of humans and other warm-blooded animals. Some of the genera are pathogenic and usually associated with intestinal infections, whereas others are essential and are part of the normal flora. Examples are the pathogenic *Salmonella*, which is a common source of food poisoning, compared with commensal non-pathogenic *Escherichia coli* strains, which have beneficial traits for humans, such as synthesizing vitamin K from undigested material in the large intestine [1]. Physical and chemical host barriers of the innate immune system generally protect the host from invading pathogens by activating macrophages, a special type of phagocyte, to engulf and destroy the invaders. Activated macrophages produce ROS (reactive oxygen species) and RNS (reactive nitrogen species), which are able to modify or inactivate proteins, lipids and nucleic acid compounds of the engulfed micro-organism, and thereby kill them [2]. One RNS that has sparked a great deal of interest in recent times is the potent cytotoxin nitric oxide (NO) that is lethal to most pathogens. NO is generated in macrophage lysozymes by iNOS (inducible nitric oxide synthase). When iNOS becomes activated, it catalyses the oxidation of L-arginine to L-citrulline and NO [3]. The generation of ROS is performed by the NADPH oxidase Phox, and genetic defects affecting this enzyme lead to an increased rate of infections in humans [4]. Phox reduces O_2 to O_2^-, which dismutates into hydrogen peroxide. The reactivity of NO and hydrogen peroxide results in the generation of other reactive compounds such as peroxynitrite. If mice lack iNOS or Phox, or both, they are much more susceptible to *Salmonella* infections, resulting in higher fatality rates and increased tissue damage of liver and spleen [5,6]. On the one hand, this underpins the importance of both enzymes for the immune system to deter invading pathogens. On the other hand, it highlights the importance of detoxification mechanisms for pathogenic bacteria such as *Salmonella*. In addition, NO is generated after a nitrate-rich meal. Dietary nitrate produces salivary nitrite, which becomes acidified in the stomach and is further converted into NO [7,8]. It has been shown that the NO levels generated in the stomach are far beyond its beneficial use as a vasodilator and that it supports the killing of pathogens in addition to the stomach acidity [7]. However, some pathogens such as *Salmonella* have evolved the ability to protect themselves against oxidative and nitrosative stresses. They are able to detoxify NO and related RNS to less toxic compounds and thereby ensure their survival. The same defence mechanisms may be used by bacteria producing NO endogenously as a

Key words: *Escherichia coli*, nitrate respiration, nitric oxide detoxification, nitrous oxide, pathogenicity, *Salmonella enterica* serovar Typhimurium.

Abbreviations used: FNR, fumarate and nitrate reductase regulator; Fur, ferric-uptake regulator; iNOS, inducible nitric oxide synthase; MetR, methionine repressor; RNS, reactive nitrogen species; ROS, reactive oxygen species; SCV, *Salmonella*-containing vacuole; SPI-2, *Salmonella* pathogenicity island 2.

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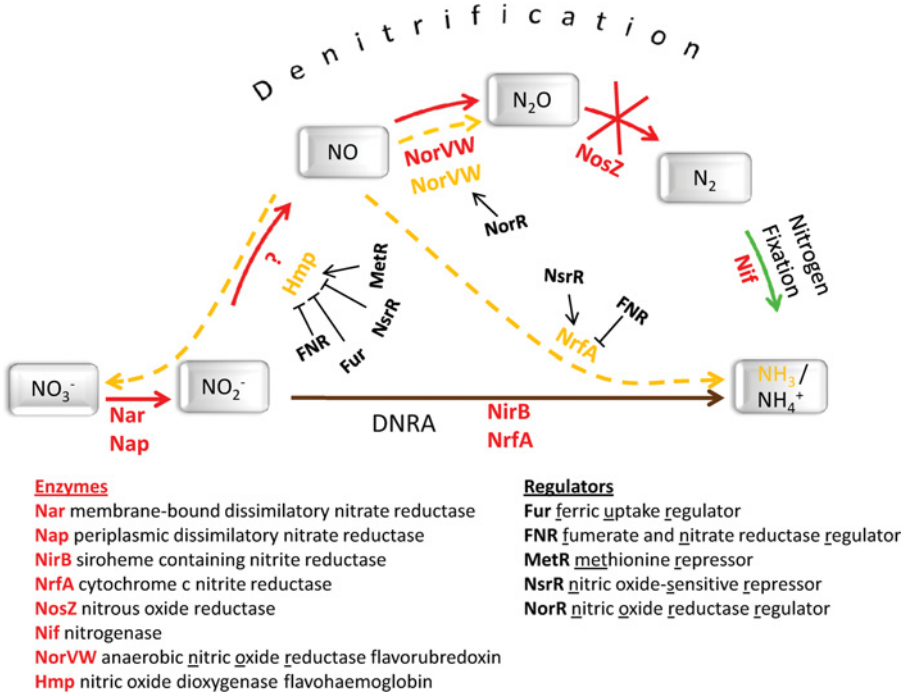
Figure 1 | **Truncated denitrification pathways in *E. coli* and *Salmonella* Typhimurium**

Nitrate respiration in *E. coli* and *Salmonella* is a truncated version of the denitrification pathway (red arrows). Unlike many soil bacteria, *E. coli* and *Salmonella* lack NosZ; indicated by a red cross. NO is a toxic intermediate. The main enzymes involved in NO detoxification alongside their regulators are shown. The NO detoxification pathways are indicated by yellow broken arrows. Enzymes involved in these pathways are shown in yellow. Positive regulation is highlighted by arrows, and negative regulation by perpendicular lines. Other enzymes are shown in red.



by-product of their own metabolism during anaerobic nitrate respiration.

NO detoxification in enteric bacteria

As *E. coli* and *Salmonella* are facultative anaerobes, O_2 is their preferred energy source, if present. However, if there is a shortage of O_2, they are able to switch to nitrate respiration to maintain their metabolism in a process called denitrification [9]. Unlike many soil bacteria, *E. coli* and *Salmonella* undergo only truncated denitrification, where the alternative electron acceptor nitrate is converted into nitrous oxide (N_2O) via nitrite and NO. The subsequent conversion of nitrous oxide into dinitrogen gas is lacking. The enzymes involved in these reactions (Figure 1) are dependent on cofactors for correct functioning, most commonly metal cofactors such as molybdenum, copper and iron–sulfur [Fe–S] clusters. The lack of N_2O reduction only makes a minor difference to the bacterium bioenergetically [9], but, on the other hand, the ability to detoxify NO is very important. Although it has been controversial for a long time whether NO itself is toxic or only the resulting RNS [10], recent studies have clearly proven that NO has cytostatic and cytotoxic effects on both aerobically and anaerobically grown cultures [11,12]. Its reactivity with [Fe–S] clusters, thiol groups and ROS results in extensive damage of DNA, proteins and transcription factors, in particular [13–15].

Enteric pathogens must have evolved mechanisms to overcome NO produced by the immune system as well as to defend themselves against their own toxic metabolites. *E. coli* and *Salmonella* are known to possess three major enzymes to perform this role. They comprise the soluble flavohaemoglobin Hmp, the di-iron-centred flavorubredoxin NorV with its NADH-dependent oxidoreductase NorW (NorVW) and the cytochrome *c* nitrite reductase NrfA [16,17]. All enzymes vary in importance under different environmental conditions. Hmp is able to cope with oxic as well as anoxic conditions, and produces nitrate and N_2O respectively. Both the NorVW and Nrf enzymes are only active under anaerobic or micro-oxic conditions [18]. NorVW reduces NO to N_2O, whereas NrfA uses either NO or nitrite to form ammonia. It has been shown that NorV and NrfA are the most important enzymes in anaerobic NO detoxification of *Salmonella* [16]. Hmp has only a minor role in NO detoxification under anoxic conditions, but it is the crucial enzyme when O_2 is present [16–18]. The combined activity of the three enzymes allows *Salmonella* and *E. coli* to be very flexible in their metabolism and hence helps them to survive in a range of different environments. This ability is also advantageous outside the host because high nitrate

concentrations and therefore high NO generation is also seen in wastewater and soil. High nitrate levels in these environments are mainly caused by manure from humans and other animals and the excessive use of nitrate-containing fertilizers.

Another reason for this high flexibility is due to various transcription factors being differently transcribed under specific conditions [19]: the main regulators that mediate a response to NO in *Salmonella* and *E. coli* include NorR, NsrR, FNR (fumarate and nitrate reductase regulator) and MetR (methionine repressor) [12].

NorR exclusively regulates the *norVW* genes in response to nitrosative stress. MetR is implicated in the regulation of *hmp* in both organisms, alongside the NO-sensitive repressor NsrR that, in addition, also regulates the expression of *nrfA* [19–21]. NsrR belongs to the Rrf2 family of transcriptional repressors and senses NO specifically by a [2Fe–2S] cluster [22]. This assumption results from great similarities between NsrR and other [2Fe–2S] cluster-containing members of the Rrf2 family such as IscR or RirA. The presence of the [Fe–S] clusters makes the protein structure and binding prone to damage by NO. It has been reported that *E. coli* genes, which are repressed by NsrR, are derepressed after exposure to NO [23]. Other regulators that are important in stress response and co-ordination of gene expression are FNR and Fur (ferric-uptake regulator) [23–25]. FNR possesses a master function in the transition between aerobic and anaerobic growth and mediates the up-regulation of several operons in response to nitrate and nitrite [25]. *Hmp* and *ytfE* are among the genes that are repressed by FNR, but the addition of either nitrite or nitrate causes an activation of the gene expression. This indicates a putative regulatory mechanism, which ensures that the expression of *hmp* will not be disabled during exposure to RNS. Exposure to NO damages the [Fe–S] clusters of FNR and results in the derepression of the protective flavohaemoglobin *hmpA* [26]. It has been demonstrated that *ytfE* plays a crucial role in the repair of NO- and ROS-damaged [Fe–S] clusters [27]. Furthermore, NO-sensitivity and growth impairment of *ytfE* mutants showed its importance in the response to oxidative and nitrosative stresses [14] and its di-iron centre has been structurally characterized [28]. Fur is also affected by the presence of NO, potentially by a reaction of the protein-bound iron with NO [23]. Fur mainly regulates genes that are involved in the uptake of iron, but it also moderately regulates *hmp* expression [12]. It has been proposed that Fur regulation becomes important once iron is limited; however, there are still controversial opinions about the repressor function of *hmp* [12].

Salmonella also utilizes SPI-2 (*Salmonella* pathogenicity island 2) for NO protection. SPI-2 encodes a TTSS (Type III secretion system), which allows formation of an SCV (*Salmonella*-containing vacuole) in the intracellular environment and prevents lysosomal fusion. This prevents the co-localization of Phox and iNOS with the SCV, hence reducing the exposure of *Salmonella* to nitrosative and oxidative stresses [29].

However, we and others believe that additional unknown pathways with important roles in NO protection remain to be characterized for both organisms [30]. In search of such mechanisms, transcriptomic analyses have proven to be helpful to highlight potential genes involved [19]. Gene annotations based on homology provide some insight into possible proteins expressed, but do not always highlight functions that are of higher physiological relevance. Therefore the function of putative NO-detoxification genes and proteins needs further investigation, particularly with respect to infection.

N_2O production in enteric bacteria

Salmonella and *E. coli* are commonly exposed to different stresses as they have various interactions with the body. This suggests that their response to stresses such as nitrosative stress and hence N_2O production varies as well. Since NO is highly reactive, it will quickly become detoxified by the conversion into nitrous oxide in the cytoplasm of *Salmonella* and *E. coli*. This process serves to convert a potent cytotoxin into a potent greenhouse gas.

Enteric bacteria can produce NO as a side product of nitrate or nitrite metabolism. One major source of this NO in *Salmonella* has been suggested to be the reduction of nitrite by the NarG nitrate reductase [31]. This endogenous NO leads to derepression of genes encoding systems that are concerned with the detoxification of NO and the repair of proteins damaged by the cytotoxin. There have been reports of nitrous oxide release by pure cultures of Enterobacteriaceae, including *E. coli*, *Klebsiella pneumoniae* and *Salmonella enterica* during nitrate metabolism that presumably reflects NO being converted into nitrous oxide [32,33]. Whether there is a physiological importance for generating and releasing the neuropharmacological agent nitrous oxide to an enteric pathogen as a side product of their nitrate metabolism has yet to be addressed, but it is an interesting question.

Concluding remarks

The significance of NO production is well studied in relation to human or murine macrophages as part of the immune defence mechanisms; however, this is not the case for the detoxification of NO and the subsequent production of N_2O by pathogens. Three enzymes have been identified that contribute significantly to NO detoxification. Therefore several questions need to be addressed in the future. Which additional mechanisms contribute to the detoxification of NO, either directly by enzymatic conversion of NO or indirectly, repairing the damage caused? Does the production of N_2O differ between pathogenic and non-pathogenic bacteria?

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Resolving the contributions of the membrane-bound and periplasmic nitrate reductase systems to nitric oxide and nitrous oxide production in *Salmonella enterica* serovar Typhimurium

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The production of cytotoxic nitric oxide (NO) and conversion into the neuropharmacological agent and potent greenhouse gas nitrous oxide (N_2O) is linked with anoxic nitrate catabolism by *Salmonella enterica* serovar Typhimurium. *Salmonella* can synthesize two types of nitrate reductase: a membrane-bound form (Nar) and a periplasmic form (Nap). Nitrate catabolism was studied under nitrate-rich and nitrate-limited conditions in chemostat cultures following transition from oxic to anoxic conditions. Intracellular NO production was reported qualitatively by assessing transcription of the NO-regulated genes encoding flavohaemoglobin (Hmp), flavorubredoxin (NorV) and hybrid cluster protein (Hcp). A more quantitative analysis of the extent of NO formation was gained by measuring production of N_2O, the end-product of anoxic NO-detoxification. Under nitrate-rich conditions, the *nar*, *nap*, *hmp*, *norV* and *hcp* genes were all induced following transition from the oxic to anoxic state, and 20 % of nitrate consumed in steady-state was released as N_2O when nitrite had accumulated to millimolar levels. The kinetics of nitrate consumption, nitrite accumulation and N_2O production were similar to those of wild-type in nitrate-sufficient cultures of a *nap* mutant. In contrast, in a *narG* mutant, the steady-state rate of N_2O production was ~30-fold lower than that of the wild-type. Under nitrate-limited conditions, *nap*, but not *nar*, was up-regulated following transition from oxic to anoxic metabolism and very little N_2O production was observed. Thus a combination of nitrate-sufficiency, nitrite accumulation and an active Nar-type nitrate reductase leads to NO and thence N_2O production, and this can account for up to 20 % of the nitrate catabolized.

Key words: Enterobacteriaceae, nitrate reductase, nitric oxide, nitrite reductase, nitrous oxide, *Salmonella*.

INTRODUCTION

The Enterobacteriaceae family of Gammaproteobacteria are found naturally in soils, water systems and sewage and as a part of the gut flora in the gastrointestinal tract. They are facultative anaerobes that can, with few exceptions, use nitrate or nitrite as terminal respiratory electron acceptors. The availability of these electron acceptors varies in the different environments to which the bacteria adapt [1]. For an enteric pathogen, for example, the gastrointestinal tract can be rich in nitrate or nitrite, but nitrate is more scarce in bodily fluids such as the bloodstream [1]. In many species of Enterobacteriaceae, there are two biochemically distinct nitrate reductases: one membrane-bound with the active site located in the cytoplasm and the other in the periplasm. These are coupled to two nitrite reductases to provide parallel pathways for nitrate reduction to ammonium in the two cellular compartments and that are differentially expressed in response to different nitrate and nitrite concentrations [1,2]. In the cytoplasm, nitrate is reduced to nitrite by a membrane-bound respiratory nitrate reductase system (NarGHI):

$$NO_3^- + 2e^- + 2H^+ \rightarrow NO_2^- + H_2O \text{ (Reaction 1)}.$$

The nitrite produced can then be reduced further to ammonium by a sirohaem-containing nitrite reductase (NirB):

$$NO_2^- + 6e^- + 8H^+ \rightarrow NH_4^+ + 2H_2O \text{ (Reaction 2)}.$$

In the periplasm, the process involves two different enzymes: a periplasmic nitrate reductase (NapA) that reduces nitrate to nitrite and a periplasmic cytochrome *c* nitrite reductase (NrfA) that further reduces the nitrite to ammonium. Reactions 1 and 2 together lead to the production of extracellular ammonium and are often termed DNRA (dissimilatory nitrate reduction to ammonium).

Salmonella and *Escherichia coli* produce the cytotoxin nitric oxide (NO) as a side-product of nitrate or nitrite metabolism [3,4]. One major source of this NO has been suggested to be the reduction of nitrite by the membrane-bound nitrate reductase NarG [3]. This endogenous NO leads to derepression of genes encoding systems that are concerned with the detoxification of NO and the repair of proteins damaged by the cytotoxin. The regulator that mediates this derepression is the NO-binding protein NsrR (NO-sensing repressor) [5–7]. A key enzyme in the NsrR regulon in *Salmonella enterica* serovar Typhimurium is flavohaemoglobin (Hmp) that reduces two molecules of NO to one molecule of nitrous oxide (N_2O) under anoxic conditions, using cytoplasmic NADH as electron donor [8,9]. This represents the conversion of a potent cytotoxin into a product that is both a neuropharmacological agent and potent greenhouse gas [10]. There have been reports of N_2O release by pure cultures

Abbreviations used: D, dilution rate; DNRA, dissimilatory nitrate reduction to ammonium; Hmp, flavohaemoglobin; Km^R, kanamycin-resistance; MS, minimal salts; NapA, periplasmic nitrate reductase; Nar, membrane-bound nitrate reductase; NirB, cytoplasmic sirohaem nitrite reductase; NorV, flavorubredoxin; NrfA, periplasmic cytochrome *c* nitrite reductase; NsrR, NO-sensing repressor; qc, specific rate of consumption; qRT, quantitative real-time; S_{BR}, bioreactor concentration of substrate; S_R, reservoir concentration.

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Table 1 All strains and plasmids used in the present study

Strain or plasmid	Genotypes or relevant characteristics	Reference
Strains		
SL1344	*Salmonella* Typhimurium, *his*$^-$, mouse-virulent	[40]
Δ*hmpA*	SL1344 Δ*hmpA*::kan	[29]
Δ*norV*	SL1344 Δ*norV*::cat	[29]
Δ*norV*Δ*hmpA*	SL1344 Δ*hmpA*::kan Δ*norV*::cat	[29]
Δ*narGHJI*	SL1344 Δ*narGHJI*::kan	The present study
Δ*napFDAGHBC*	SL1344 Δ*napFDAGHBC*::kan	The present study
Δ*nirB*	SL1344 Δ*nirB*::kan	The present study
Δ*hcp*	SI1344 Δ*hcp*::kan	The present study
Plasmids		
pKD4	Ap^R, pANT-Sγ derivative containing an FRT-flanked Km^R	[17]
pKD46	Ap^R, pINT-ts derivative containing *araC*-P$_{araB}$ and γ, β, *exo* genes	[17]

of Enterobacteriaceae, including *Escherichia coli*, *Klebsiella pneumoniae* and *S. enterica* during nitrate metabolism, and it is likely that this is due to reductive detoxification of NO produced as a side-product of nitrate metabolism [11,12,18–20]. In the present paper, we describe a biochemical study on nitrate catabolism in continuous cultures of the food-borne pathogen *Salmonella* Typhimurium which reveals that intracellular NO production and associated extracellular N_2O production can account for up to 20 % of nitrate catabolized and is linked to the culture nitrate status, nitrite accumulation from nitrate respiration and the biochemical type of the nitrate reductase system expressed.

EXPERIMENTAL

Bacterial strains and growth media

Salmonella Typhimurium strains (Table 1) were cultivated anaerobically in MS (minimal salts) medium [13]. The sole carbon and electron source was glycerol, the sole terminal electron acceptor was sodium nitrate, with ammonium (15 mM) present as a nitrogen source. Continuous culture was performed in a New Brunswick Scientific BioFlo 3000 fermenter with a 1.2 litre working volume under pH control (pH 7.0, 1 M NaOH and 1 M HCl/0.1 M H_2SO_4 used for regulation). A 100 ml volume of MS medium was inoculated with 5 ml of an overnight culture and aerobically incubated overnight at 37 °C. Then, 50 ml of this culture was used to inoculate the bioreactor. After 24 h of aerobic batch growth, the air supply was switched off and a feed of MS medium was started to achieve a dilution rate (D) of 0.04 h^{-1}. The measured dissolved O_2 (percentage air saturation) in the culture fell from 100 % to 0 % within 1 h of switching off the air supply and was monitored throughout the continuous culture phase of the experiment to ensure that it remained at 0 %. During the experiment, samples were taken at regular intervals to determine the attenuance at 600 nm (D_{600}), protein concentration and nitrogen compound composition.

Protein and nitrogen compound quantification

Protein concentration was determined using the method described in [14]. Nitrate was determined via HPLC using the anion-exchange column Ion Pac AS22, 2 mm×250 mm (Dionex, ICS-900) as described by the manufacturer. Nitrite was measured colorimetrically with a modified Griess reaction [15]. A PerkinElmer Clarus® 500 Gas Chromatograph with an ECD (Electron Capture Detector) and Elite-PLOT Q (DVB Plot Column, 30 m length; 0.53 mm internal diameter; carrier gas, N_2; make-up gas, 95 % argon/5 % methane; temperatures as described by the manufacturer) were used with known gas standards of N_2O [0.4, 100, 1000 p.p.m., supplied from StGas] to determine the N_2O concentration in headspace gas samples. From this, the total N_2O in headspace and solution was calculated by applying Henry's Law, assuming equilibration between the solution and gas phases and using a Henry's Law constant at 37 °C of 0.453.

Enzyme activity

The NarGH nitrate reductase complex, comprising the 140000 Da NarG and the 60000 Da NarH subunits, was purified from anaerobic cultures of *Salmonella* Typhimurium and assayed using Methyl Viologen as the electron donor, essentially as described previously for *Paracoccus pantotrophus* NarGH [16].

RNA extraction, qRT (quantitative real-time)-PCR and mutant construction

RNA was extracted at the appropriate time points from nitrate-sufficient and -limited continuous cultures of *Salmonella* Typhimurium SL1344 using a Promega SV 96 total RNA purification kit. The total RNAs were first treated with Turbo DNaseFree from Ambion and the absence of DNA contamination was verified by PCR. RNA quality was assessed on an Agilent 2100 Bioanalyser. Then, 2 μg of DNaseI-treated total RNA were retro-transcribed from random hexamers (Invitrogen) with Superscript II RT (Invitrogen) according to the manufacturer's recommendations. Specific primers for the genes of interest amplifying an average product of 100 bp with an approximate T_m (melting temperature) of 60 °C were designed. The qRT-PCRs were performed on a 5-fold dilution of the total cDNA obtained, using the Bio-Rad Laboratories CFX96 instrument and SensiMix™ SYBR No-ROX kit (Bioline). The qRT-PCR experiments were performed in triplicate, with three independent total RNA preparations. The calculated threshold cycle (C_t) for each gene was normalized to the C_t of the *ampD* gene, expression of which is invariant across a large range of growth conditions. Mutants were constructed in *Salmonella* Typhimurium SL1344 using λ Red mutagenesis [17]. Oligonucleotides were designed which deleted the entire gene or operon in question inclusive of start and stop codons. A linear PCR product was generated using a template plasmid, pKD4, resulting in a Km^R (kanamycin-resistance) gene cassette with 40 bp of homologous sequence flanking to the loci. The amplified DNA fragment was column-purified and electroporated into *Salmonella* Typhimurium SL1344pKD46. Mutations were confirmed by PCR using primers external to the site of mutagenesis; as well as internal

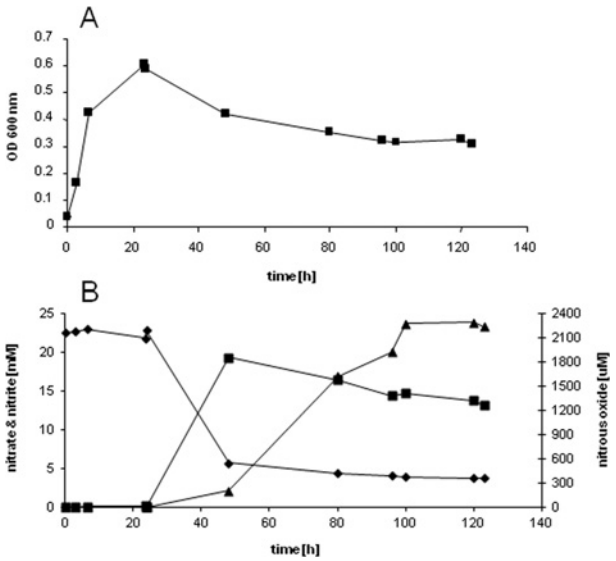

Figure 1 Nitrate, nitrite and N_2O consumption or production in a glycerol-limited nitrate-sufficient continuous culture of *Salmonella* Typhimurium SL1344

The culture was initially grown in batch mode under atmospheric oxygen concentration for 24 h, during which time cellular biomass [indicated by an increase in D_{600} ('OD')] was generated. The air supply to the culture was then switched off and the system switched to continuous mode at a dilution rate of 0.04 h^{-1}. The measured dissolved O_2 (percentage air saturation) in the culture fell from 100 % to 0 % within 1 h of switching off the air supply, and was monitored throughout the experiment to ensure it remained at 0 %. The pH and temperature were maintained at 7 and 37 °C respectively. The glycerol concentration in the reservoir feed was 5 mM and the nitrate concentration was 22.5 mM. (**A**) Biomass; (**B**) nitrate (◆), nitrite (■) and N_2O (▲). For clarity, only the results from a single chemostat run are shown. The results (± S.D.) derived from replicate experiments is given in Table 2.

primers within the Km^R cassette, k1 and k2 [17]. P22 transduction was used to transfer the mutations into a clean SL1344 background.

RESULTS

Nitrate respiration and N_2O production in nitrate-sufficient continuous cultures

Salmonella Typhimurium was cultured to anoxic steady-state in continuous cultures with nitrate (reservoir concentration, S_R, 22.5 mM) present as the respiratory electron acceptor and glycerol (S_R 5 mM) present as the carbon source for anabolism and electron source for respiration. The cultures were grown in batch mode under an atmospheric oxygen concentration for 24 h, during which time cellular biomass (x) was generated (Figure 1A). The air supply to the culture was then switched off and the system was switched to continuous mode ($D = 0.04\ h^{-1}$). There was a decrease in D_{600} and protein concentration in the bioreactor as the culture shifted from aerobic to anaerobic metabolism, until a new biomass steady-state was reached after three or four bioreactor vessel volume changes (~80–120 h). During the transitional, non-steady-state, phase (between 24 and ~80 h) the nitrate concentration in the bioreactor ($S_{BR}NO_3^-$) decreased, consistent with a shift from oxygen respiration to anaerobic nitrate respiration (Figure 1B). The shift to nitrate respiration was also reflected by increased transcription of both the *narG* and *napA* nitrate reductase genes and the gene for the nitrate/nitrite antiporter *narK*, detected using qRT-PCR analysis, at 80 h and 120 h compared with 5 h (Figure 2).

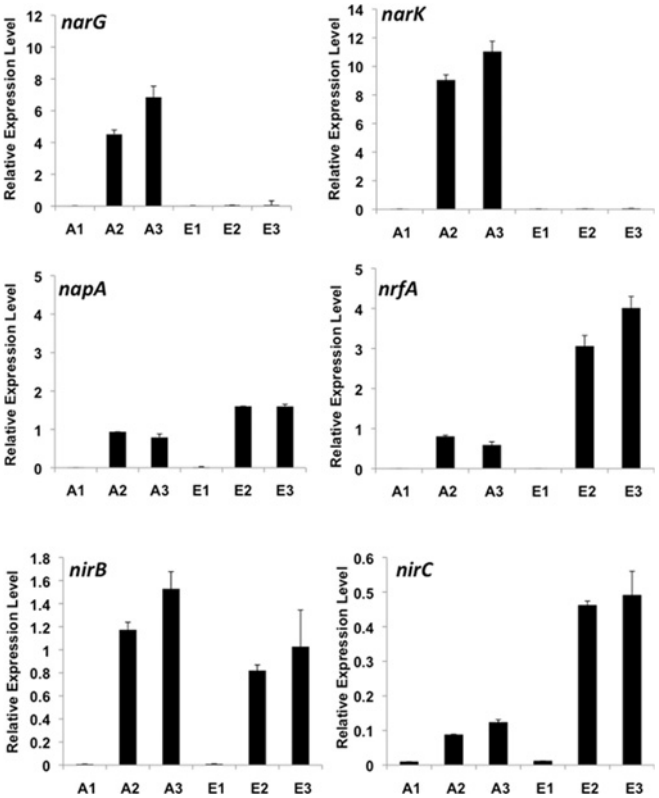

Figure 2 qRT-PCR of genes involved in nitrate and nitrite transport and reduction

The qRT-PCR experiments were performed in triplicate, with three independent total RNA preparations. The calculated threshold cycle (C_t) for each gene was normalized to the C_t of the *ampD* control. Results are means ± S.D. A, nitrate sufficiency; E, nitrate limitation; 1, 5 h oxic; 2, 80 h anoxic; 3, 120 h anoxic.

Nitrite was produced almost stoichiometrically with nitrate consumption during the first ~20 h of the transition phase (24–48 h) (Figure 1B). This is consistent with nitrite being produced directly from nitrate reduction by the nitrate reductase(s) (Reaction 1), such that the $S_{BR}NO_3^-$ decreased from ~22 mM to ~5 mM and the $S_{BR}NO_2^-$ increased from ~0 mM to ~18 mM (Figure 1B). Over the next 50 h, the $S_{BR}NO_2^-$ decreased by ~4.5 mM from ~18 mM to a steady-state value of ~14 mM (100–120 h). In steady state, the specific rate of nitrate consumption ($qcNO_3^-$) was ~20 % higher than the specific rate of nitrite accumulation ($qpNO_2^-$) (Table 2). To account for this difference some of the nitrite produced from nitrate in Reaction 1 must be further consumed by the culture. This rate of consumption ($qcNO_2^-$) can be estimated from the difference between $qcNO_3^-$ and $qpNO_2^-$ (Table 2). Ammonium is a possible net product of nitrite reduction by the NirB or NrfA nitrite reductases (Reaction 2), with *nirB* in particular being strongly induced during the anaerobic phase (Figure 2). However, the $S_{BR}NH_4^+$ remained constant at ~13 mM throughout the continuous culture phase of the experiment, which represented a net consumption of ~2 mM of the 15 mM NH_4^+ in the reservoir feed for anabolic purposes.

NO is also a potential net product of nitrite reduction, but extracellular accumulation was not detected above 1 μM. However, increased transcription of *hmp*, *hcp* and *norV* was observed following the transition from oxic (5 h sampling time) to anoxic (80 and 100 h sampling times) conditions (Figure 3). These genes are regulated by the cytoplasmic NO-responsive transcription factors NsrR (*hmp* and *hcp*) and NorR (*norV*),

Table 2 The steady-state rates of nitrate, nitrite and N_2O production in chemostat cultures of *Salmonella* Typhimurium

The data presented are taken during steady state at 100–120 h from replicate continuous cultures run in parallel under identical conditions. $qcNO_3^- = (S_RNO_3^- - S_{BR}NO_3^-)D/x$; $qpNO_2^- = (S_{BR}NO_2^-)D/x$; $qcNO_2^- = qcNO_3^- - qpNO_2^-$; $qpN_2O = (S_{BR}N_2O)D/x$. N_2O data are expressed as nitrogen-equivalents to allow for direct comparison between N_2O and nitrate or nitrite. ND, not detectable, nitrite detection limit = 0.005 mM.

Strain	NO_3^-	Glycerol	Biomass ($g \cdot l^{-1}$)	NO_3^- consumed ($mmol \cdot l^{-1}$)	NO_2^- produced ($mmol \cdot l^{-1}$)	N_2O produced ($mmol \cdot l^{-1}$)	$qcNO_3^-$ ($mmol \cdot g^{-1} \cdot h^{-1}$)	$qpNO_2^-$ ($mmol \cdot g^{-1} \cdot h^{-1}$)	$qcNO_2^-$	qpN_2O ($mmol \cdot g^{-1} \cdot h^{-1}$) [1)]
SL1344	22.5	5	0.19 ± 0.01	18.1 ± 0.7	14.0 ± 0.3	4.0 ± 0.2	3.81 ± 0.19	2.95 ± 0.15	0.86	0.850 ± 0.050
SL1344	5.5	22	0.18 ± 0.01	5.0 ± 0.5	ND	0.016 ± 0.003	1.11 ± 0.10	ND	1.11	0.004 ± 0.001
nar	22.5	5	0.11 ± 0.01	16.0 ± 0.5	16.5 ± 1.0	0.076 ± 0.004	6.0 ± 0.6	6.0 ± 0.6	0	0.028 ± 0.003
nap	22.5	5	0.15 ± 0.02	21.0 ± 0.2	15 ± 1.0	3.6 ± 0.4	5.6 ± 1.0	4.0 ± 0.5	1.60	0.96 ± 0.10

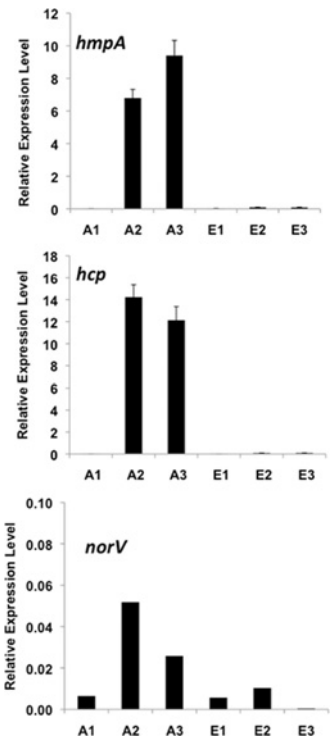

Figure 3 qRT-PCR of genes regulated by the NO-responsive transcription factors NsrR and NorR

The qRT-PCR experiments were performed in triplicate, with three independent total RNA preparations. The calculated threshold cycle (C_t) for each gene was normalized to the C_t of the *ampD* control. Results are means ± S.D. A, nitrate sufficiency; E, nitrate limitation; 1, 5 h oxic; 2, 80 h anoxic; 3, 120 h anoxic.

and so this increased transcription is indicative of intracellular NO production. It was notable that N_2O accumulated as the $S_{BR}NO_2^-$ decreased in the transition phase between 48 and 100 h (Figure 1B). The steady-state rate of N_2O production (qpN_2O), when normalized for the two nitrogens in N_2O compared with the one nitrogen in NO_2^-, matched the steady-state rate of nitrite consumption (Table 2), showing that this N_2O production is closely linked to the metabolism of nitrite generated from nitrate metabolism. Since *hmp*, *hcp* and *norV* transcription indicated the production of intracellular NO, then the series of reactions that lead to 2 mol of NO_2^- being reduced to 1 mol of N_2O via NO is predicted to be:

$$2[NO_2^- + 2H^+ + e^- \rightarrow NO + H_2O] \text{ (Reaction 3)}$$

$$2NO + 2H^+ + 2e^- \rightarrow N_2O + H_2O \text{ (Reaction 4)}$$

Reaction 4 is known to be catalysed by both the Hmp and NorV (flavorubredoxin) enzymes under anoxic conditions, and so *hmp* and *norV* mutants were also analysed under the nitrate-sufficient continuous culture conditions. In the case of strains carrying single lesions in either *hmp* or *hcp*, the rate of N_2O production in steady state was comparable with wild-type. However, a double *hmp nor* mutant only produced N_2O at ~40 % of the rate of the wild-type, suggesting functional overlap of these two systems in NO detoxification and N_2O production under anoxic conditions. In total, ~18 mM nitrate in the feed reservoir was consumed in the steady state ($S_RNO_3^- - S_{BR}NO_3^-$ at $t = 120$ h) and ~4 mM nitrogen equivalents of N_2O was produced (Table 2). This represents a conversion of ~20 % of nitrate into N_2O (Table 2).

Comparison of N_2O production in *Salmonella* Typhimurium *nar* and *nap* mutants in nitrate-sufficient continuous cultures

The NarG nitrate reductase has previously been implicated in both NO and N_2O production and nitrosation in Enterobacteriaceae [2,3,11,12,18–20]. However, a number of Enterobacteriaceae species only have a Nap type of nitrate reductase [2], and, in those that have both Nar and Nap, the nitrate-rich growth conditions under which many previous studies of NO or N_2O production has been made would not be those that favour *nap* expression, which is maximal under nitrate-limiting conditions [1,2,5,21–23]. Thus the question of whether activity of Nap can lead to NO and N_2O production has not been directly addressed before. To investigate this, isogenic *narG* and *napA* strains were constructed. Both were able to grow under anaerobic conditions with nitrate as a sole electron acceptor, but a double *narG napA* mutant could not. Under the nitrate-rich continuous culture conditions, the *nar* strain, which is dependent on Nap for growth, achieved a steady-state anoxic biomass of ~60 % of the wild-type strain under identical culture conditions (Figure 4A and Table 2). The kinetics of nitrate consumption and nitrite accumulation during the aerobic–anaerobic transition phase were closely matched (Figure 4B and Table 2), suggesting that the respiratory nitrite reductase systems Nrf or Nir (Reaction 2) do not operate at a significant level to consume the nitrite produced from Reaction 1. The rate of nitrate consumption in the steady state (100–120 h) was comparable with that of the wild-type (Table 2). Significantly, however, the steady-state $S_{BR}N_2O$ was only ~0.08 mM in the *nar* strain (Figure 4B and Table 2), such that the steady-state rate of N_2O production (qpN_2O) was approximately 30-fold lower than for the wild-type strain (Table 2). In contrast, the rate of N_2O production in the *nap* strain was comparable with that of

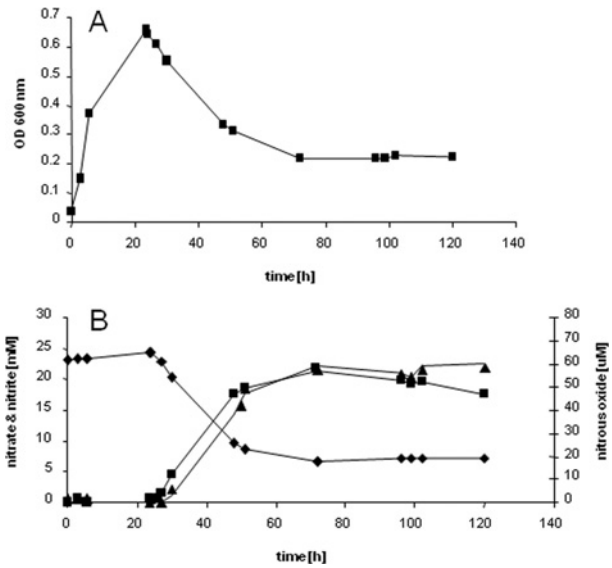

Figure 4 Nitrate, nitrite and N_2O consumption or production in a glycerol-limited nitrate-sufficient continuous culture of an *Salmonella* Typhimurium mutant deficient in Nar

The culture was run as described for Figure 1. The glycerol concentration in the reservoir feed was 5 mM and nitrate concentration was 22 mM. (**A**) Biomass; (**B**) nitrate (◆), nitrite (■) and N_2O (▲). For clarity, only the results from a single chemostat run are shown. The results ($\pm$S.D.) derived from replicate experiments is given in Table 2.

the wild-type (Table 2). The large difference in the rate of N_2O production between the *nap* and *nar* strains (Table 2) suggests that it is not associated with respiration in nitrate-rich cultures conditions itself, but with metabolism via the Nar system, rather than the Nap system, under nitrate-rich culture conditions.

NarG generates nitrite in the cytoplasm and it has been argued, on the basis of genetic and microbiological data, that this can compete with nitrate for the Nar active site in the cytoplasm [3], with Nar then catalysing Reaction 3. To demonstrate biochemically that purified *Salmonella* Nar can reduce nitrite, we isolated the membrane-associated NarGH complex from detergent-solubilized membranes of *Salmonella* Typhimurium. The enzyme complex displayed nitrate and nitrite reductase activities that each obeyed a Michaelis–Menten-type dependency on substrate concentration (Figure 5). These activities were fully sensitive to low concentrations of azide (20 μM), which is a potent inhibitor of Nar-type nitrate reductases and NO production and nitrosation by Enterobacteriacaea [19,24]. The K_m and V_{max} values for nitrate reduction were determined as 123 ± 14 μM and 83 ± 10 μmol $\cdot$ min^{-1} $\cdot$ mg^{-1} respectively (Figure 5A). For nitrite reduction, the K_m and V_{max} values were determined as 5200 ± 1900 μM and 24 ± 5 μmol $\cdot$ min^{-1} $\cdot$ mg^{-1} respectively (Figure 5B). Using a molecular mass for NarGH of 200 000 Da, the k_{cat} values for nitrate and nitrite could be approximated as 270 s^{-1} and 80 s^{-1} respectively.

In addition to Nar reducing nitrite to NO, it is possible that nitrite generated in the cytoplasm by Nar is a substrate for another NO-generating nitrite-reducing enzyme. The likely candidate for this would be the cytoplasmic NADH-dependent nitrite reductase NirB, which was also induced following transition to anoxic steady state (Figure 2), and has been implicated previously in cytoplasmic NO generation [25]. A *nirB* strain was therefore constructed and grown in the nitrate-rich continuous cultures, but the behaviour was identical with that of wild-type, with the specific rate of N_2O production was similar to that of wild-type.

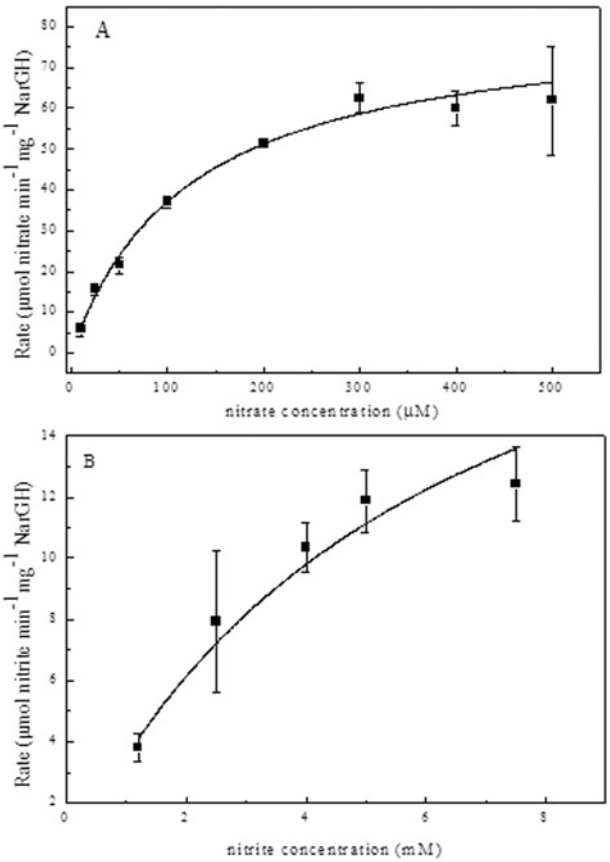

Figure 5 The kinetics of nitrate (A) nitrite (B) reduction by NarGH from *Salmonella* Typhimurium

Assays were undertaken in 20 mM Hepes and 2 mM EDTA (pH 7.0) under anaerobic conditions in nitrogen-sparged sealed cuvettes using reduced Methyl Viologen as electron donor. The data are fitted to the Michaelis–Menten kinetic model with the K_m and V_{max} values for nitrate reduction as 123 ± 14 μM and 82 ± 10 μmol $\cdot$ min^{-1} $\cdot$ mg^{-1} respectively, and K_m and V_{max} values for nitrite reduction as 5200 ± 1900 μM and 24 ± 5 μmol $\cdot$ min^{-1} $\cdot$ mg^{-1} respectively.

The NrfA nitrite reductase system has been shown previously to play a role in detoxifying NO added exogenously to *Salmonella* and *E. coli* [26–30]. Some reports have suggested that a product of NO metabolism by Nrf can be N_2O [31]. To assess this, a *nrfA* periplasmic nitrite reductase mutant was also examined in nitrate-rich continuous culture, but, like the *nirB* mutant, it behaved identically with wild-type. Thus, although *nrfA* was expressed in the anoxic phase of the cultures (Figure 2), the periplasmic NrfA enzyme does not appear to be important for detoxification of endogenously produced intracellular NO.

Nitrate metabolism in nitrate-limited continuous cultures

The net consumption of $\sim$17 mM nitrate and 5 mM glycerol by the nitrate-sufficient cultures suggested that running the continuous cultures with a $S_R NO_3^-$ of $<$17 mM and a $S_R$glycerol of $>$5 mM would lead to nitrate limitation. To achieve this condition, the $S_R NO_3^-$ was lowered to 5.5 mM and the $S_R$glycerol concentration increased to 22 mM (Figure 6A). Under these conditions, the $S_{BR} NO_3^-$ was very low under steady-state conditions (100–120 h) (Figure 6B), consistent with nitrate limitation. qRT-PCR revealed up-regulation of *nap*, but not *narG*, following the oxic–anoxic transition, consistent with Nap being the enzyme of choice for metabolism under nitrate-limited

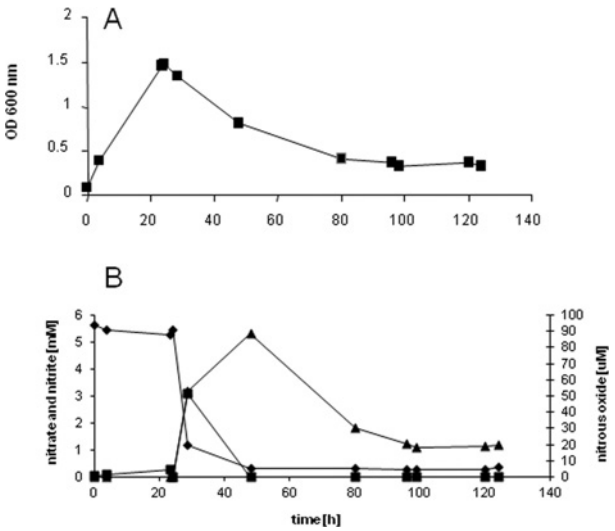

Figure 6 Nitrate, nitrite and N_2O consumption or production in a glycerol-sufficient nitrate-limited continuous culture of *Salmonella* Typhimurium SL1344

The culture was run as described for Figure 1. The glycerol concentration in the reservoir feed was 22 mM and nitrate concentration was 5 mM. (**A**) biomass; (**B**) nitrate (◆), nitrite (■) and N_2O (▲). Results are illustrative for experiments run in triplicate. For clarity, only the results from a single chemostat run are shown. The results ($\pm$ S.D.) derived from replicate experiments are given in Table 2.

conditions (Figure 2). During the transition phase of 24–48 h, nitrite accumulated in the reactor vessel to a maximum of $\sim$3 mM, concomitantly with nitrate consumption. However, this was only transient, and nitrite was not detectable (detection limit of 0.005 mM) when the cultures reached steady state (Figure 6B). Thus the rates of nitrate and nitrite consumption (Reactions 1 and 2) were matched (Table 2). Significantly, the profile of N_2O accumulation was quite different from the nitrate-sufficient cultures. Like nitrite, N_2O accumulated transiently, and the peak of production lagged approximately 20 h behind that of nitrite, but the maximum obtained was 20-fold lower than the maximum obtained in the nitrate-sufficient cultures (compare Figure 6B with Figure 1B). In steady state (100–120 h), there was only minimal N_2O release, the rate of which was more than two orders of magnitude lower than for the nitrate-sufficient cultures (Figure 6B and Table 2), with less than 0.1 % of the nitrate-nitrogen ending up as N_2O.

DISCUSSION

In the present study, we have examined nitrate catabolism and associated exogenous N_2O production during continuous culture of *Salmonella* Typhimurium under nitrate-rich and nitrate-limited anoxic conditions. NO is detoxified by conversion into N_2O in the cytoplasm (Reaction 4), and, as a consequence, the direct measurement of NO released by bacteria will grossly underestimate the actual level produced intracellularly during nitrate metabolism. *Salmonella* Typhimurium cannot reduce N_2O and so measuring its extracellular release is a good quantitative measure for the fraction of nitrate catabolized that forms NO intracellularly. This has been illustrated in the present study where, under nitrate-rich conditions, millimolar levels of extracellular N_2O were measured, but extracellular NO was not detected above 1 μM. This demonstrates that, under the steady-state metabolic conditions established, there is highly efficient reductive detoxification of endogenously produced cytotoxic NO to N_2O, which then escapes from the cell. From this, it can be estimated that up to 20 % of nitrate catabolized is converted into the NO, which represents a substantial scale of intracellular production and thus requires very efficient detoxification of the cytotoxin.

N_2O production was maximal under nitrate-rich culture conditions where both *nar* and *nap* were expressed. However, mutagenesis confirmed that Nar was the major enzymatic route for the nitrate catabolism associated with N_2O production. (Figure 4 and Table 2). In the 20 h following anoxia, nitrate was consumed and nitrite accumulated in a near-stoichiometric fashion and very little N_2O accumulated (Figure 1B). The likely biochemical processes in operation are first nitrate import via the nitrate/nitrite antiporter NarK [32], the gene for which was also up-regulated during nitrate catabolism under nitrate-sufficient conditions. This is then followed by reduction to nitrite by NarG, and export of nitrite by NarK in exchange for incoming nitrate (Figure 7A). This process generates protonmotive force for growth. It is notable that, under these nitrate-rich culture conditions, the consumption of the nitrite produced from nitrate respiration is minimal and there is extensive extracellular accumulation of nitrite rather than the further reduction to ammonium (Figure 1B) despite expression of both *nirB* and *nrfA* (Figure 7A). Thus, although *Salmonella* Typhimurium is considered to be a canonical DNRA organism, under these nitrate-rich conditions, nitrite, rather than ammonium, is the major extracellular end-product of nitrate respiration. It makes sense from a bioenergetic view point to maximize nitrate respiration and minimize nitrite respiration under electron acceptor-sufficient conditions. This is because the $\uparrow H^+/e^-$ stoichiometry for reduction of nitrite by NrfA (2 with NADH as electron donor and 0 with quinol as electron donor) is lower than for nitrate reduction by Nar (3 with NADH and 1 with quinol) [33]. In this respect, it also makes bioenergetic sense to utilize the NarG nitrate reductase system, rather than the Nap system, since the $\uparrow H^+/e^-$ coupling ratios for periplasmic reduction of nitrate by Nap (2 with NADH and 0 with quinol) are also less than for Nar. This is highlighted by the lower biomass yield when the mutant in *narG*, that is dependent on Nap for growth, was cultured under the nitrate-sufficient conditions (Table 2).

At the end of the transition phase of the anoxic nitrate-sufficient continuous culture, the nitrite reaches an extracellular level that is $\sim$3-fold higher than that of nitrate (Figure 1). It is under these conditions that the rate of N_2O production is maximal. The rate of N_2O production matched the rate of nitrite reduction and the levels produced accounted for the balance of nitrate-nitrogen that did not accumulate as nitrite-nitrogen. The very low rate of N_2O production in the *narG* mutant suggested that it was linked to nitrite reduction by NarG. Competition between two substrates (nitrite and nitrate) for a single active site can be described by:

$$v_{nitrite}/v_{nitrate} = (k_{cat}/K_m)_{nitrite}/(k_{cat}/K_m)_{nitrate} \times ([nitrite]/[nitrate]) \text{ (adapted from [34])}$$

From the steady-state chemostat fluxes (Table 2), $v_{nitrite}/v_{nitrate} = qcNO_2^-/qcNO_3^- = 0.226$. From the kinetic parameters derived from the purified NarGH, $(k_{cat}/K_m)_{nitrite}/(k_{cat}/K_m)_{nitrate} = 0.007$. Thus [nitrite]/[nitrate] = 32 and this equates to the steady-state intracellular ratio of the two substrates. Such a ratio is perfectly conceivable if some of the nitrite exported by NarK in exchange for incoming nitrate re-enters the cell, possibly via by the bidirectional nitrite channel NirC [31], the gene for which was expressed under these growth conditions (Figures 2

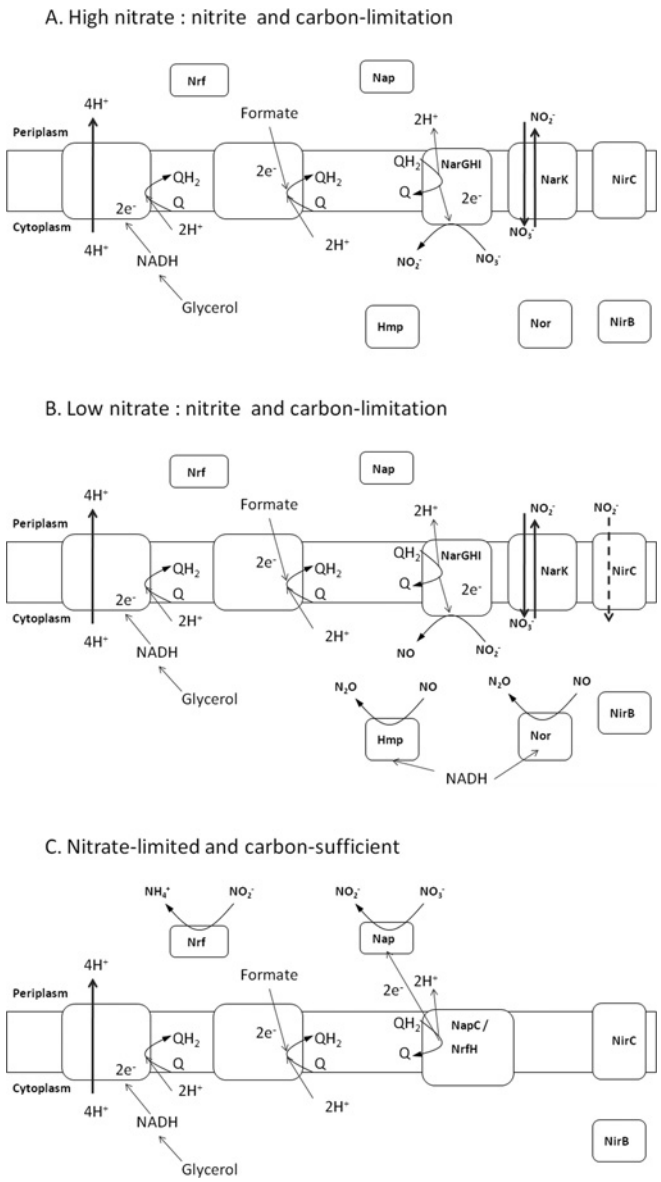

Figure 7 Schemes for nitrate and nitrite metabolism in *Salmonella* Typhimurium SL1344 under the different growth conditions explored in the present study

(**A**) In electron-acceptor-rich high-nitrate/nitrite conditions. Nar reduces nitrate to nitrite which is exported via NarK to the periplasm where it accumulates almost stoichiometrically with the nitrate consumed. (**B**) In electron-acceptor-rich low-nitrate/nitrate ratios, nitrite is imported by NirC and consumed via Nar producing NO, which is detoxified by Hmp and NorV to produce N_2O. (**C**) Under nitrate-limiting growth conditions, Nar is not synthesized, and the Nap and Nrf systems are actively consuming nitrate and the nitrite produced from nitrate reduction (with electrons flowing via the NapC and NrfH quinol dehydrogenases). No intracellular NO is generated, *hmp* and *norV* expression is low and N_2O is not produced.

and 7B). Intracellular nitrite may then out-compete intracellular nitrate for the active site of a NarG. This argument is supported by the experiments with the NarG mutant under nitrate-sufficient conditions that showed a near-stoichiometric consumption of nitrate and production of nitrite, and a very low level of N_2O production in the steady state (Figure 4). It is also consistent with the data of the early 1980s that led to the conclusion that NarG might reduce nitrite to N_2O and with more recent genetic and microbiological data that suggested that NarG in fact reduces nitrite to NO [3,11,12,18–20].

Under nitrate-limited growth conditions, the production of N_2O was very low in the steady-state phase, where both nitrate and nitrite were only present at low-micromolar levels. These conditions promoted expression of *napA* and *nrfA*, but not *narG* (Figure 2). This is consistent with the expression pattern observed in *E. coli* under low-nitrate/nitrite growth conditions [22,23,35–37]. Under these conditions, the absence of nitrite accumulation reflects that it is fully reduced through to ammonium by NrfA (Figure 7C, Reaction 2), so that there is maximum utilization of the limited respiratory electron-acceptor pool available to the culture. Thus results of the present study for *Salmonella* Typhimurium confirm observations from *E. coli* that when nitrate is sufficiently abundant, the bacteria exploit the energy-efficient, but low-affinity, NarG enzyme to reduce nitrate in the cytoplasm [22,23,35–37]. When nitrate is scarce, Nap provides a higher-affinity, but more poorly coupled, pathway that does not require nitrate transport for nitrate to serve as an effective electron sink [21] (Figure 7C).

Broadening the implications of the results more widely for the many species of Enterobacteriaceae that synthesize both NarG and NapA, the primary role of NarG is to generate protonmotive force when nitrate is abundant, such as occurs in nitrate-rich carbon-limited soils and sediments or wastewater-treatment plants [2]. Under these conditions, it has previously been thought that nitrite reductase NirB protects the cytoplasm from nitrite toxicity. However, the results of the present study show that nitrite can accumulate to millimolar levels in the extracellular medium and that, in fact, what nitrite is consumed is reduced to N_2O (Reactions 3 and 4) not ammonium (Reaction 2). However, in many habitats, Enterobacteriacaea will encounter much lower concentrations of nitrate, where the periplasmic pathway for nitrate and nitrite reduction is active [2]. We have shown that this combination will not lead to intracellular NO production, as judged by the lack of N_2O production and the absence of up-regulation of the *hmp* and *hcp* genes of NsrR regulon. The comparative experiments conducted with the *narG* and *napA* mutants demonstrate that nitrate reduction by Nap does not lead to extensive N_2O production even under nitrate-sufficient conditions.

Comparison of the *narG* and *napA* strains revealed a higher cell yield using Nar rather than Nap, reflecting the higher $\uparrow H^+/e^-$ for quinol oxidation by nitrate for Nar (1) compared with Nap (0) [33]. When Nap is operational, growth is dependent on energy-conserving formate dehydrogenase and NADH dehydrogenase reactions associated with glycerol metabolism for energy conservation, with *nap* serving a quinol pool recycling role [33]. However, although it makes bioenergetic sense to use NarG, under nitrate-rich conditions, there is a downside, which is the risk of cell damage associated with cytotoxic NO production. Co-ordinate induction of a NO-detoxification system minimizes this risk. There is, however, an energetic cost in using electrons in the non-energy-conserving cytoplasmic reduction of NO. The overall reduction of nitrite to N_2O via NarG and then HmpA or NorV using NADH consumes 2 mol of NADH (4 mol of e^-; Reactions 3 and 4) and yields an overall $\uparrow H^+/e^-$ of 1.5, which compares with an overall $\uparrow H^+/e^-$ of 2 when NADH is used via the respiratory electron-transport chain to reduce nitrite to ammonium. Thus it merits reflection on whether the diversion of $\sim$20 % of nitrate into NO and thence N_2O has any physiological importance. N_2O is a neuropharmacological agent that inhibits a range of cell receptors and transporters. The significance of N_2O production by pathogens has not been addressed, but it is likely to be a property of many enterobacterial pathogens and also pathogens such as *Neisseria* that have truncated denitrification pathways in which N_2O is the product of nitrite reduction due to the lack of N_2O reductase [38,39]. Further studies on the significance of N_2O production by pathogenic bacteria are therefore merited.

AUTHOR CONTRIBUTION

Daniela Hensen designed and executed the continuous culture experiments. Heather Felgate and Anke Arkenberg contributed to the continuous culture experiments. Corinne Appia-Ayme undertook the qRT-PCR experiments. Karen Prior and Carl Harrington constructed some of the mutants. Sarah Field undertook the enzyme kinetics. Julea Butt and Elizabeth Baggs contributed to the experimental concepts and discussion of the results. Gary Rowley and David Richardson designed the experiments and wrote the paper.

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